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(57) Abstract

The present invention relates to hepatitis B virus (HBV) disease and in particular provides polynucleotide sequences which are characteristic of certain disease states. Said HBV polynucleotides are for use in evaluation of a HBV disease state which comprises at least two of the following: (i) a mutation in the Enhancer I region; (ii) a mutation in the Negative Regulatory Element region; (iii) a mutation in the Enhancer II/Core Upstream Regulatory Sequence/Basal Core Promoter region; and (iv) a mutation which leads to an X peptide amino acid change to provide a cysteine or methionine residue; the mutation being a variation from the normal nucleotide at that position in a respective one of HBV genotypes A to F. The invention also provides tests for detecting the polynucleotides. A test involving protein binding interactions with the polynucleotides is also provided.

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HEPATITIS B VIRUS MUTATIONS

The present invention relates to hepatitis B virus (HBV) disease, and in-particular provides polynucleotide sequences which are characteristic of certain disease states. The invention also provides tests for detecting the polynucleotides. A test involving protein binding interactions with the polynucleotides is also provided.

Fulminant hepatitis B [FHBV] is a rare but serious complication of acute infection, associated with massive hepatocellular necrosis and mortality rates exceeding 50%, depending on coma grade [1,2,3]. FHBV pathogenesis has been associated with elevated rates of viral replication [4,5] and/or abnormally rapid clearance.

There has been considerable debate as to the relative role of host versus viral factors in the aetiology of FHBV. Outbreaks have been traced to common sources [5,6,7,8], suggesting the involvement of specific viral strains. Furthermore, particular viral variants have been implicated, such as the precore stop variant A_{1896} [5,6,9,10], which inhibits production of HBeAg, a protein believed to have immunomodulatory effects [11,12,13] and which may exert a dominant negative effect on HBV replication [14]. An association has also been found between FHBV and variants in the basal core promoter (BCP)[15](Figure 1), which regulates the production of precore and pregenomic RNAs; in particular, T_{1762} and A_{1764} [16,17] have been implicated. However, as with A_{1896}, these variants have also been found in non-FHBV sequences and do not occur in all cases of FHBV [18,19,20]. Complicating the issue, index case and contact pairs have been identified where the complete viral genomes are identical, or nearly so [21,22].One study showed division of patients with FHB into separate classes - rapid (<1-2 weeks) and slow (> 3weeks) - according to rates of clearance of HBsAg and HBV DNA and intervals between onset of clinical illness and hepatic encephalopathy [23].

We have tested the hypothesis that there are common patterns of genetic variation or 'motifs' among FHBV strains . Using statistical and phylogenetic analyses of the X and C genes from unrelated FHBV cases and contacts, of which 19 are previously unreported sequences, and sequence information from enhancer I region, we show that there are strains of HBV associated with this disease, each with a distinct variant pattern, effect on mortality and speed of progression to FHBV symptoms.

Thus, we have reviewed HBV sequences associated with fulminant hepatitis disease in a number of patients. We have been unable to associate any single mutation with the fulminant form of the disease. According to the present invention, by phylogenetic analysis we have surprisingly found that certain combinations of unusual nucleotide substitutions (not only relative to the known

respective genotype but which are sometimes also unusual in all genotypes) in particular positions in the sequence correlate strongly with FHBV disease. The mutations may not in themselves be novel, but in combination in the respective genotype background they are important. It also appears that a variety of mutation combinations can lead to a common phenotype. The combinations of particular nucleotides in certain positions are referred to as "motifs" herein.

The present invention in one aspect provides hepatitis B virus polynucleotide for use in evaluation of a hepatitis B disease state which comprises at least two of the following:

(i) a mutation in the Enhancer I region;

(ii) a mutation in the Negative Regulatory Element region;

(iii) a mutation in the Enhancer II/Core Upstream Regulatory Sequence/Basal Core Promoter region; and

(iv) a mutation which leads to an X peptide amino acid change to provide a cysteine or methionine residue;

the mutation being a variation from the normal nucleotide at that position in a respective one of HBV genotypes A to F. There may be one or more mutations in any of (i) to (iv).

The hepatitis disease state which may be evaluated according to the present invention include fulminant hepatitis, severe chronic hepatitis, and serologically unusual forms of hepatitis (including HBsAg negative infection, anti-HBcore negative infection and serologically negative hepatitis - all of which have been linked to variation in the X peptide or the other regions involved in transcriptional control as set out above).

Another aspect of the invention provides hepatitis B virus polynucleotide for use in detection of fulminant hepatitis B viral infection which comprises at least two of the following:

(i) a mutation at one or more of the following positions 1050, 1249 and 1250;

(ii) a mutation at one or more of the following positions 1633 and 1634;

(iii) a mutation at one or more of the following positions 1653, 1754, 1762, 1764, 1766, 1768, 1809, 1821, 1826 and 1838/9 insertion;

(iv) a mutation which leads to an X-peptide change to provide cysteine or methionine at one or more of amino acid positions 26, 72, 88, 127 and 130;

the mutations being variations from the normal nucleotide at that postion in a respective one of HBV genotypes A to F. On transmission to a contact these sequences may result in fulminant hepatitis B.

The polynucleotide sequences may be DNA or RNA sequences. Sequences are given in the 5' to 3' direction. Sequence numbering is according to the convention established by Galibert, and

numbers from the unique ECoR1 restriction site of HBV. The polynucleotide sequence need not be continuous and may be broken up into contiguous or non-contiguous segments. However, the segments will, of course, have a common origin.

The polynucleotides and peptides useful for the present invention are generally in isolated form, isolated from materials from the host patient, and usually isolated from other material of HBV origin.

Generally, the polynucleotides are fragments of the complete genome e.g. 20-1000 nucleotides in length, particularly 50-500, especially 100-200. The term 'fragment' means a polynucleotide of length less than the corresponding HBV genotype genome. X peptide fragments are usually less than the length of the naturally occurring gene expression product.

Certain polynucleotides and peptides are novel and these form a further aspect of the invention.

The hepatitis disease state may be determined directly by sequencing an HBV sample from the patient. More conveniently, the polynucleotide motifs may be detected using hybridisation probes of complementary sequence which are capable of binding thereto under appropriate stringency conditions (as may be determined readily by experimentation). Generally each probe will be directed to a portion of the sequence containing a single mutation. However, probes directed against multiple mutations may also be used. The probes may be labelled with conventional labels, such as radiolabels, fluorescence labels etc. A particular test format comprises a solid substrate having thereon a series of bands of characteristic hybridisation probes. The pattern of binding of an HBV sample from a patient to the bands enables a determination of the presence or absence of motifs characteristic of a particular disease state to be made.

Another test format for the detection of polynucleotide motifs characteristic of FHBV involves conducting polymerase chain reaction (PCR) using primers having a sequence complementary to the FHBV characteristic sequences given above.

We have confirmed that certain nucleotide motifs in the NRE/BCP/Enhancer II region (a region having gene expression control properties) lead to enhanced gene expression in an experimental plasmid (containing a luciferase gene under control of said motif-containing HBV enhancer region). Enhanced expression and replication of HBV virus in vivo is postulated to lead to the acute fulminant HBV disease state.

Without wishing to be bound by theory, it is hypothesised that enhanced gene expression resulting from the motif-containing sequences is due to a reduction of the ability of the motif-

containing control sequences to bind inhibitory proteins present in host cells (i.e. the patient). Normal inhibition of HBV gene expression is thus reduced. Experimental evidence of altered nucleotide-host protein binding is given herein.

Thus, the invention also provides a test method for determining binding interaction between host or viral proteins and HBV regulatory polynucleotides.

The invention also provides certain antigenic X-peptides, antibodies (e.g. monoclonal) thereto, and immunoassays.

Embodiments of the invention will now be described by way of example only. Sections I and II are devoted to phylogenetic analysis and increased transcription respectively.

SECTION I

Statistical and phylogenetic analysis was carried out as follows, to show that certain mutational motifs were related to FHBV.

METHODS

Patients and Sequence Data

Because previous studies have shown that sequences from index and chronic contacts of FHBV patients are often genetically identical [21, 22], all chronic [CHBV] and acute [AHBV] contact cases were treated as FHBV. For the C gene (nucleotide positions 1814-2458) viral sequences from 30 patients linked to FHBV, representing 27 unlinked FHBV episodes, were analysed. Of these 30, 19 (16 FHBV, 2 CHBV, 1 AHBV) are newly reported here and 11 (6 FHBV, 5 CHBV) were obtained from the GenBank/EMBL/DDBJ sequence databases. For the X gene (nucleotide positions 1374-1838) 26 FHBV sequences representing 25 unlinked FHBV episodes were analysed. Of these 26, 17 (14 FHBV, 2 CHBV, 1 AHBV) are newly reported here and 9 (6 FHBV, 3 CHBV) were obtained from the sequence databases. With respect to the non-FHBV sequences, 2 X and C gene sequences are newly reported here while 159 complete C gene sequences and 86 complete X gene sequences were obtained from the sequence databases or from a recent study [19] (sequence list available from authors). This produced total datasets of 191 C gene and 114 X gene sequences. All unpublished sequences have been submitted to GenBank and have been assigned accession numbers xxxx - yyyy.

Fulminant patient information is detailed in Table 1. Patients were classified as either 'rapid' or 'slow' progressors to FHBV symptoms (hepatic encephalopathy). Rapid progressors were those who progressed from stage 0 to stage 4 coma within a week. Sequences from contact cases first

reported here include two symptomless anti-HBe positive carriers (sequences 10 and 21) who fatally infected two sexual partners each [6], and one of a pair of sisters, infected simultaneously, one of whom developed mild acute infection (sequence 7) while the other acquired severe FHBV (sequence 6). 2 patients were serum anti-HDV IgM positive and thus co-infected with hepatitis D virus. None of our patients were positive for anti-HCV antibodies. In order to assess the incidence of variation in enhancer I a GenBank/ncbi/BLAST search identified all published sequences in the region (74 sequences) (list available from authors).

DNA extraction and PCR amplification

DNA extraction, PCR amplification and sequencing were performed as previously described [27] (list of primers available from authors).

Phylogenetic Analysis

C and X gene sequences were aligned with the ClustalW program [28] and phylogenetic trees constructed using a maximum likelihood method (program fastDNAml [29]). The maximum likelihood transition:transversion ratio (Ts/Tv) and relative rates of evolution for the three codon positions were estimated using a likelihood program (SPOT) [30]. The parameters so determined are listed in the figure legends for Figures 2 and 3. In all cases unrooted phylogenetic trees were constructed and then midpoint rooted for clarity. For technical reasons, there was insufficient FHBV sequence information available for an analysis of FHBV sequence clustering in the enhancer I region.

Relative Rates of Evolution

In order to determine whether there was any difference in the rate of evolution between FHBV and non-FHBV sequences, a relative rate test was undertaken (Figure 4). Distances between FHBV sequences (*a*) and their nearest non-FHBV neighbor (*b*) were compared to their nearest non-FHBV outgroup sequence (*c*) using the DNADIST (nucleotide) and PROTDIST (amino acid) programs within the PHYLIP package [31]. 13 and 14 comparisons were made for the X and C genes, respectively. Tests were also performed for the antigenic regions in the C gene as defined in Carman et al. 1995 [32] and according to the functional subdivisions in X identified in Yuh et al. 1992 [33]. Analysis at the protein level was limited statistically to the complete X and C genes and the combined antigenic and combined non-antigenic regions in C. Relative rate tests were also performed on FHBV sequences with and without the A_{1896} pre-core stop codon variant (9 and 7 comparisons, respectively) and on a set of non-FHBV A_{1896} cases (9 comparisons). In the tests involving A_{1896}, sequences from G_{1896} (HBeAg producing) CHBV cases acted as sequences *b* and *c*. In all cases the Wilcoxon Signed Rank Test was used to test for the significance of any differences in evolutionary rate.

Finally, variants were divided into those that altered the encoded amino acid (nonsynonymous changes) and those that did not (synonymous changes) and a comparison made between FHBV and non-FHBV cases using the program INA[34] . The relative rate of synonymous and nonsynonymous change is a useful indicator of the strength of natural selection.

Analysis of Clustering

A cluster was defined here as an uninterrupted phylogenetic lineage of epidemiologically unlinked FHBV sequences. We undertook an analysis to determine whether FHBV sequences form distinct clusters on the tree more than expected by chance. This was done by treating FHBV status as an additional character state and calculating the expected number of evolutionary steps given the number of FHBV cases under a parsimony based model of evolutionary change [35], as implemented in the program FMAB (Bollyky et al., submitted for publication).

RESULTS

Is there genetic similarity between epidemiologically unlinked FHBV cases? While visual inspection of the X gene (Figure 2) and C gene (Figure 3) phylogenetic trees suggests that FHBV sequences are clustered, it was necessary to prove this statistically. For the X gene, the distribution of the 25 independent FHBV sequences on the phylogenetic tree could be accounted for most parsimoniously by 14 unambiguous evolutionary steps, fewer than the minimum of 21 expected under a random model of sequence evolution ($P = 0.1 \times 10^{-5}$). In the C gene, the 17 observed unambiguous evolutionary steps for the 27 independent FHBV sequences was again less than the 25 expected if these sequences were not clustered ($P = 0.4 \times 10^{-7}$). This clustering reflects a high degree of genetic relatedness amongst FHBV sequences. Generally the same clusters were found in both genes, although sequences 15, 19, 21, 23 occupied different positions on the X and C gene trees. Whether these discrepancies reflect recombination events [36], as was clear in the case of non-clustered FHBV sequences 25 and 26, or the effect of rapid evolution associated here with FHBV sequences (see below), was unclear.

Do the clusters segregate according to sex, age, HBeAg status, viral genotype, or clinical outcome of their hosts? Table 1 details these associations. Clustering was most strongly linked to mortality; every cluster of sequences being uniform with respect to outcome (i.e. survival or death) with 2 exceptions, one was a liver transplant recipient and thus survived while the other was pregnant and died. All FHBV cases in individuals who were pregnant, hepatitis D virus co-infected, or over 30 years of age were fatal, with the exception of those patients who received liver transplants. Clustering was also linked to the speed of onset of hepatic encephalopathy and clearance of HBsAg and HBV DNA, with every cluster of sequences, with 2 exceptions, being uniform in this regard. The two patients who were co-infected with hepatitis D virus were found in the same cluster.

Are FHBV sequences characterised by different rates of nucleotide evolution and by changes in specific regions? Relative to their nearest non-FHBV phylogenetic neighbor, the FHBV sequences had a significantly higher rate of nucleotide evolution in both the X and C genes (Tables 2 and 3 respectively). In the X gene, this increase was also significant at the amino acid level, and localised to the BCP at both the nucleotide and amino acid levels. FHBV sequences also had a significantly higher number of synonymous changes in the X gene, indicating an elevated rate of background mutation. This relationship is also reflected in the codon position specific weighting ratios determined for the X gene phylogenetic tree, where first and second codon position changes occur at an elevated rate relative to third position changes. In the C gene, the elevated rate of evolution in FHBV

sequences was significant at the nucleotide level for the whole gene and for non-antigenic regions.

Are particular variants associated with FHBV sequences and clusters of sequences ? No single unique variant linked all individual FHBV sequences or groups of sequences; this has been reported previously [15, 20, 24]. However, particular variants characterised FHBV sequences and clusters of sequences (detailed in Table 4). On the nucleotide level, these included variants in enhancer I (enhancer I is known to stimulate transcriptional function of both the X gene promoter and the BCP [37]), variants in the negative regulatory element (NRE) [38], and the BCP region of enhancer II. On the amino acid level, these included uncharacteristic patterns of otherwise highly conserved cysteine residues [39], which would be expected to play a central role in determining the tertiary structure of protein products of X, and aberrant methionine residues, which might be expected to alter levels of the three protein products thought to be coded by the X gene [40] and thus have effects on transcriptional transactivation of HBV.

Many of these nucleotide and protein variants are genotype specific, altering nucleotides otherwise typical for particular genotypes; these relationships are detailed in Table 5. Among variants which have been previously suggested to play a role in FHBV pathogenesis, T_{1676} and A_{1757}, [15] were found to be characteristic of genotype D; 30 and 21 of the 35 genotype D sequences in our dataset possessed these variants, respectively, and so they were not associated with FHBV.

Were there particular 'motifs', or combinations of variants, which were unique to FHBV? The FHBV sequences possess particular motifs, or combinations of variants which distinguish them from non-FHBV sequences. The combination of either aberrant methionine residues, aberrant cysteine residues, or one of three identified nucleotide variants in enhancer I with any of a group of notable variants in the NRE, the BCP, or the A_{1896} pre-core stop variant was nearly exclusive to FHBV sequences (Table 6). T_{1762} and A_{1764} [16, 17] characterised some clusters of FHBV sequences but were not by themselves disproportionately represented in FHBV cases. However, where both these two variants are present with either T_{1766} or A_{1768}, 3 of 4 examples were FHBV. With a single exception, whenever the A_{1896} variant was found with the aberrant cysteine and methionine residues or enhancer I variants described here it was in a sequence associated with FHBV.

What effect does the A_{1896} precore stop variant have on the rate and pattern of evolution in FHBV sequences? In G_{1896} FHBV sequences and in the complete FHBV dataset (A_{1896} plus G_{1896} FHBV sequences), changes in the complete C gene and in non-antigenic regions accumulated faster than in non-FHBV sequences. In contrast, A_{1896} FHBV sequences had significantly higher rates of nucleotide evolution than in G_{1896} non-FHBV sequences in the complete C gene and in both non-

antigenic and antigenic regions, particularly within the anti-HBc / e2 and anti-HBc / e3 epitopes, as well as significantly higher rates of amino acid evolution in all areas tested. These results for A_{1896} FHBV also differed from the CHBV A_{1896} controls, which did not accumulate changes at a significantly increased rate in non-antigenic regions and differed in the specific antigenic regions affected (Table 3).

TABLE LEGENDS.

TABLE 1:Clinical data and clustering of FHBV cases and contacts.

A cluster was defined as an uninterrupted phylogenetic lineage of epidemiologically unlinked FHBV sequences. Occasionally there where minor discrepancies in the clusters defined in the X and C genes due to lack of phylogenetic resolution. In these cases, such as the movement of sequence 19 between clusters 1 and 6, weight was given to the positioning seen within the X gene, as this is the gene where the majority of the variants associated with FHBV are described. Those sequences which do not cluster in the X gene but do in the C gene, either because those sequences were unavailable for the X gene or because of the factors such as recombination or the rapid evolution associated here with FHBV, are marked with a *. Sequences 25 and 26 are epidiomologically linked and therefore are not numbered as a cluster. Individual sequences are those which do not cluster with other fulminant sequences in either gene.

C,X= Core and X genes. M=Male,F=Female.FHBV= Fulminant hepatitis B virus infection; AHBV= Acute hepatitis B virus infection; CHBV = Chronic hepatitis B virus infection.[5] Reported here. **For chronic contacts, the outcome and course listed are those of the fulminant index cases associated with that contact case.@ Patient was pregnant.

± Patient recieved liver transplant. β Patient was hepatitis D virus co-infected. § These sequences show evidence of recombination, grouping with D genotype sequences in the C gene and with A genotype sequences in the X gene.

TABLE 2: Relative rate tests for the X gene comparing rates of evolution between FHBV and non-FHBV sequences.

All results are expressed as *P*= the probability that FHBV sequences are evolving faster than non-FHBV sequences. Significant results are in bold type.* Nucleotides are numbered from the unique

EcoR1 site.± Amino acids are numbered from the start of the X gene. CURS= Core Upstream Regulatory Sequence, BCP= Basal Core Promoter. Relative rate test results for synonymous changes were significantly different only for the number of synonymous changes in the X gene between FHBV and non-FHBV sequences (*P*=0.014).

TABLE 3:

Relative rates tests for the C gene comparing rates of evolution in the complete FHBV dataset, A_{1896} FHBV variants, G_{1896} FHBV variants, and non-FHBV A_{1896} variants, all against G_{1896} non-FHBV sequences.

All results are expressed as *P*= the probality that FHBV sequences are evolving faster than non-FHBV sequences. Significant results are in bold type. * Nucleotides are numbered from the unique EcoR1 site. Relative rate tests for synonymous and nonsynonymous changes were not significantly different between FHBV and non-FHBV sequences in any areas tested.

TABLE 4: Variants suggested to play a role in FHBV and their distribution in the dataset.

BCP= Basal Core Promoter, NRE= Negative Regulatory Element, Pre-C = Precore region; A_{1896}= premature stop codon that inhibits HbeAg production.

*There were a number of variants in the BCP/enhancer II complex whose incidence was unique to a single sequence or pair of sequences. These were: A_{1779}(CHBV1), A_{1790}(CHBV1), A_{1794}(CHBV1), T_{1810}(HPBC5HK02, HPBC4HST2), T_{1811}(HPBC5HK02,HPBC4HST2), C_{1819}(FHBV13), C_{1821}(CHBV1), and A_{1826}(FHBV1).

± Variation related to FHBV at these sites involves deviations from otherwise highly conserved genotype nucleotide identity. More than one nucleotide variant therefore describes the relevant variation at this position (Table 5). § Where applicable. += That variant seen, -= Other variant seen at that position, NA= Not available.**Sequences FHBV15 and CHBV1 have insertions at position 1838 which cause a frame shift.

TABLE 5: Combinations of variants in motifs that may play role in FHBV cases.

Variants or combinations of variants playing role in FHBV. + = variants observed. - = variants not observed. n/a = not available. ?= not known. Underlined variants are unique variants observed in that particular case or only few cases. Bold variants occurred outside the genotype context. C_{1740} is not a unique variant but significant in combination with another unusual variant (c_{1773}). *= For case

FHBV-10, no variants were identified and for case FHBV-11, sequence data was not available in the regions considered except pre-core. Enh=enhancer. BCP= basal core promoter. NRE=negative regulatory element. CURS= core upstream regulatory element.

TABLE 6: Deviations from typical genotype associations at a number of particular nucleotide and amino acid positions in the X gene.

* All three occurrences of C_{1773} in genotype A are in FHBV sequences. Variation in the other genotype at this location seems to be less restrictive.** Sequences either have a cysteine a position 6 or position 78, depending on genotype. The cysteine at position 78 has been shown to be disulphide bonded[30], suggesting this difference is of some importance in determining protein structure.

TABLE 7: Motifs, or combinations of variants, suggested to play a role in FHBV and their distribution in the dataset.

MOTIF 1= BCP nucleotide variant+ aberrant cysteine residue in X protein.

MOTIF2 = BCP nucleotide variant + aberrant methionine residue in X protein.

MOTIF 3 = NRE nucleotide variant + aberrant cysteine residue in X protein.

MOTIF 4 = NRE nucleotide variant + aberrant methionine residue in X protein.

MOTIF 5 = NRE nucleotide variant + enhancer I nucleotide variant.

MOTIF 6 = A_{1896} precore stop variant + aberrant cysteine residue in X protein.

MOTIF 7 = A_{1896} precore stop variant + aberrant methionine residue in X protein.

MOTIF 8 = A_{1896} precore stop variant + enhancer I nucleotide variant.

BCP = Basal Core Promoter, NRE= Negative Regulatory Element, Pre-C= Precore region; A_{1896}= premature stop codon that inhibits HbeAg production. Only sequences for which complete information was available for the areas in question are listed. The A_{1896} total is out of 43 X gene sequences with the A_{1896} precore stop variant.

TABLE 8: FHBV Variant Motifs

* See section on genotype specific FHBV variants.

TABLE 9: Presence or absence of variants identified in table 4 in 7 non-FHBV and 15 FHBV sequences, all of genotype D.

TABLE 10: Clinical data of FHBV and chronic control cases.

M= male, F= female, N/A= not applicable, n/D= not done, +ve= positive, -ve= negative, ++= highly positive, IV = intravenous needle

FHBV= fulminant HBV, CHBV= chronic HBV, dot/blot= DNA was detected by dot-blot hybridisation, ?= not known. *=Titre indicates final dilution at which sample remained positive.

TABLE 11: Primers used for PCR

Table 11 shows the sequences of primers in 5' to 3' direction used for PCR. The underlined sequences are the restriction sites. Outer= primers used for 1st round PCR, Inner= primers used for nested PCR.

TABLE 12: Sense strand oligonucleotides used for gel shift.

Table 12 shows the oligonucleotide sequences used for gel shift analysis. Only sense strands are shown here. Nucleotide positions of 5' and 3' ends, numbered from the Eco RI site, are given above oligonucleotide sequences.

TABLE 13: Luciferase value correlated with variation in the CURS, BCP and pre core region.

Variants found in the region between 1549-1974, which contains the CURS and the BCP/Enh-II complex, are considered. The clustering pattern and patie number follows that in the previous study (Bollyky *et al*, 1997, submitted). CURS = core upstream regulatory sequence, BCP= basal core promoter. Enh-I Enhancer II. CHBV-1 and -2 are infective contacts of fulminant cases. H= HIGH (luciferase value > 7), I= intermediate (luciferase value 2.0-7.0), N/A= No applicable, N=Normal (luciferase value < 2), Δ= deletion; $_{1838}A_{1839}$ and $_{1838}AC_{1839}$ denote an insertion of A or AC between the noted nucleotide positions. The standard deviation is derived from at least 6 replicates for each construct. Rapid/slow disease progression was a clinical judgement; allocations to categories were made before these studies were undertaken. Underlined variants are not unique but significant in comination with another variant(s).

DISCUSSION

We report the clustering of FHBV sequences from epidemiologically unlinked sequences within particular viral lineages. Further, these viral cluster relationships seemed to predict death or survival in the absence of additional confounding factors for poor prognosis such as pregnancy, coinfection with HDV and older age. This is conclusive evidence of a viral genetic basis to FHBV.

Further, 21 of the 26 X gene FHBV sequences had a combination of variants in the complex which regulates pregenomic and precore production (the NRE, the BCP region of enhancer II and the A_{1896} precore variant), plus one of 3 alterations which might be expected to affect the production or function of X gene protein products (aberrant methionine or cysteine residues or enhancer I variants). These combinations, or motifs, were nearly exclusive to FHBV sequences. We propose a novel concept in studies of HBV: individually, many of these variants are not unique to FHBV sequences but that their pathogenic significance lies in their interaction.

We hypothesise that these motifs are associated with common functional effects. The significantly higher rates of nucleotide evolution in the C and X genes and in the HBV genome as a whole (results to be published separately) suggest that an increased rate of evolution is a hallmark of FHBV. The elevated rate of synonymous changes in X and of evolution in non-antigenic regions in C are consistent with this interpretation. Further, we have shown that the BCP region of A_{1896}-associated FHBV sequences has significantly increased transcription efficiency (Yasmin et al., submitted). This supports one report of increased replication and encapsidation efficiency shown for an individual FHBV sequence [5]. Another novel, though related, concept to arise from this work is that specific variants can be linked to a different clinical outcome if they occur outside of their usual genotype context. Further, particular genotypes may predispose patients to FHBV: 15 of the 26 X gene FHBV sequences were of genotype D (out of 35 sequences available for that genotype).

These motifs, however, are clearly not entirely equivalent; the particular variants which characterise individual clusters of FHBV sequences appear to result in their distinct clinical features. A fatal outcome was associated with every FHBV sequence with either the T_{1762} or A_{1764} variants. In two other studies, 3 of 4 [18] and 4 of 5 [17] such cases were fatal. In a recent computer modelling study, these variants have been suggested to lead to alterations in RNA superstructure encompassing the encapsidation signal loop from which DNA replication is initiated [42] and are part of the TATA complex involved in initiation of precore and pregenomic transcripts [43]. Along with positions 1762 and 1764, position 1773 is part of the TATA complex. Every FHBV case associated with aberrant C_{1773} was similarly fatal. Different classes of FHBV are also associated with distinct selective forces,

as the A_{1896} FHBV sequences are shown here to have different patterns of evolution in the C gene than FHBV sequences without this variant.

The A_{1896} variant has been found in both CHBV as well as FHVB cases and it has previously suggested that the transmission of the A_{1896} variant and HBeAg negative status results in FHBV pathogenesis [5,6,10]. Building on our recent report of the transmission of the A_{1896} variant, and presumably HBeAg negative status, on a significant scale (Bollyky, et al. submitted for publication), here we conclusively show that the combination of A_{1896} with other FHBV associated variants distinguishes A_{1896} chronic from A_{1896} fulminant cases Lending further support to this hypothesis of non-equivalent effects of A_{1896} in FHBV and CHBV cases, we have also shown here that A_{1896} FHBV sequences have different patterns of evolutionary change than A_{1896} CHBV sequences.

The phylogenetic clustering of unlinked FHBV cases, when FHBV is itself a viral transmission dead end, would suggest that certain viral genetic contexts, such as those defined by strain and genotype identity, might either be particularly susceptible to acquiring specific FHBV associated variants or already possess some elements of the FHBV motif combinations. We propose that the symptomless contact who transmits HBV resulting in fulminant hepatitis has a heterogeneous population of HBV strains, or quasispecies characteristic of quickly evolving viruses [44], some of which possess all the necessary elements required to cause FHBV in *de novo* infections by triggering a massive immune response in the presence of elevated rates of viral replication.

It is clear that looking for a single mutational cause for all cases of FHBV is too simplistic. The results presented here, based on a large dataset and the application of phylogenetic and statistical methods, conclusively show that there are multiple sequence routes to FHBV pathogenesis which we believe share common functional features. While we do not discount that transmission related effects, co-infection with other viruses, and host factors such as HLA, as shown to be related to viral clearance [45], may be involved, we believe that viral sequence is a necessary prerequisite and central to the causation of fulminant hepatitis B. Greater consideration must certainly be given to the interactions of multiple viral factors and the context within which specific variants occur. Closer examination of the multiple routes leading to FHBV promises to shed light on HBV immunomodulation and transcriptional and translational control.

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FIGURES 1 to 6

Figure 1

Organisation of genes and regulatory regions along the HBV genome. CURS = core upstream regulatory region. BCP = basal core promoter. NRE = negative regulatory element. Pre-C = pre-core gene. C = core gene. X = X gene. DR = direct repeat (DR1 initiates reverse transcription, DR2 second strand synthesis). The DNA is separated into two strands for clarity (though both strands are the coding strand). The ruler indicates nucleotide positions numbered from the unique EcoR1 site.

Figure 2

Clustering of FHBV sequences on the maximum likelihood tree of the X gene. For the 191 C gene sequences the Ts/Tv was determined to be 1.24 with the relative rates of evolution as 0.87:0.62:1.51 at codon positions 1, 2 and 3, respectively. Lineages which possess FHBV status are indicated. Horizontal branch lengths are drawn to scale. Clusters of sequences, as defined as FHBV lineages which have no intervening non-FHBV sequences, are numbered in bold. Genotype of each major branch is given. Genotype E is indistinguishable from genotype D within the X gene, as has been noted previously 19.

Figure 3

Clustering of FHBV sequences on the maximum likelihood tree of the C gene. For the 114 X gene sequences the Ts/Tv ratio and the codon-specific relative rates of evolution were found to be 1.28 and 1.49:1.04:0.50 at codon positions 1, 2 and 3, respectively. Lineages which possess FHBV status are indicated. Horizontal branch lengths are drawn to scale. Clusters of sequences, as defined as FHBV lineages which have no intervening non-FHBV sequences, are numbered in bold. Genotype of each major branch is given.

Figure 4

A relative rate test compares rates of evolution in two phylogenetically related viruses, *a* and *b*, by comparing their DNA and amino acid sequences to that of a third, reference virus *c*, which is more distantly related to both (i.e. it is an outgroup). Because *a* and *b* share more recent common ancestry with respect to sequence *c*, they should be equally distant to this outgroup sequence if they are evolving at the same rate.

Figure 5

The complete DNA sequence data from 1000 to 2500 for seven non-FHBV patients (comparison) and fifteen FHBV patients (invention), all genotype D, showing the mutations from the normal at various variant positions.

Figure 6

Diagramatic representation of various mutational motifs associated with FHBV.

SECTION II

Here we show that the *cis*-acting sequences from four of the clusters have increased transcriptional activity *in vitro* and that the variant promoter sequences have lost nuclear factor binding activity.

PATIENTS AND METHODS

Cases and controls

In the phylogenetic analysis (Section I), 16 FHB cases, 3 FHB contact cases and 2 AHB cases were studied. In the functional analysis reported here, we chose 11 FHB or FHB contact cases from that study and 7 additional chronic hepatitis (CHB) controls not associated with FHB following transmission. Clinical and demographic details are given in Table 10. We grouped contacts with FHB having shown previously that their HBV sequences cluster phylogenetically. Figure 7 gives the sequences of all the subjects from the beginning of the core upstream regulatory sequence (CURS) to the end of enhancer II (nt 1643- 1849) indicating the variants we defined previously as being more common in FHB than in chronic cases, taking into account the genotype-specific variability. We have included two additional laboratory standard control sequences of subtypes *adw2* (genotype A) and *ayw* (genotype D).

DNA extraction, PCR, sequencing and sequence analysis

In brief, 25 ul serum was digested overnight in 1mg/ml proteinase K at 37° C. DNA was extracted once each with phenol/chloroform (1:1), once with chloroform and precipitated in ethanol. Finally, the DNA was resuspended in 15 ul dH_2O. For PCR amplification, 5 ul extracted DNA was amplified by 25 pmol each of sense and antisense outer primers. 1 ul of first round product was nested, or hemi-nested. A list of primers is given in Table 11. DNA was sequenced directly using

Sequenase version 2.0 (USB, Amersham, UK).

Luciferase assay

Constructs were based on vector pBL (23), which includes the luciferase gene but without its own complete promoter. Nucleotides 1549-1975 of HBV (generated by PCR using primers C8m & BC3, Table 11), which includes the negative regulatory elements (NRE), CURS, BCP, enhancer II (EnhII) and a portion of core gene were cloned into pBL upstream of the luciferase gene. Clones were sequenced to verify their identity. Luciferase assays were performed as previously described (24). In brief, 10ug plasmid DNA was transfected into two 60 mm plates containing 50-70% confluent Huh7 hepatocyte cells by standard calcium phosphate transfection. To overcome the effect of variable transfection efficiency, each plate was co-transfected with 0.2 ug pSV vector containing the β-galactosidase gene. 72 hrs after transfection, cells were lysed with 25mM Tris phosphate, 10% glycerol and 0.1% triton X-100. 50 ul of cell lysate was mixed with 350 ul of assay buffer (20mM tricine, 1.07mM $MgCO_3$-$5H_20$, 2.67mM $MgSO_4$, 0.1mM EDTA, 33.3mM DTT, 270mM Co-enzyme A, 470mM luciferin, 530mM ATP. pH 7.8), and luciferase production was either measured in an automated luminometer, or with the Luciferase assay kit (Promega, Madison, USA) following the manufacturer's instructions. For each construct, the assay was done at least 3 times; for each assay, duplicate plates were used; and for each plate, duplicate measurements were taken. The β-galactosidase assay was performed with the same cell extract. 50 ul of cell extract was incubated with a buffer (50mM Na_2HPO_4, 39mM NaH_2PO_4, 1mM HCl, 1mM $MgSO_4$, 1mg/ml ONPG) at 37°C until a pale yellow colour developed. The OD at 405 nm was measured. The luciferase value for each experiment was calculated by dividing the mean unadjusted luciferase value by the mean β-gal value and the luciferase value for each construct was the mean of all experiments.

Preparation of nuclear extract and mobility shift analyses

Nuclear extracts were made from HeLa cells and HuH7 cells by a method described by Trautwein et al (25), and stored at -70° until used; for each experiment, a fresh aliquot was used. Complementary oligonucleotide pairs corresponding to the variant HBV sequences in Enh-I and Enh-II/BCP complex (Table 3) were made either using an in-house oligo-synthesizer or purchased from Oligold (Eurogentec, Seraing, Belgium). Oligonucleotides were annealed to make double-stranded DNA was labelled with ^{32}P γATP. Labelled oligonucleotide was purified from unincorporated ^{32}P with Sephadex G-50 spin column (Pharmacia, Uppsala, Sweden). For gel shift experiments, 3-5 x 10^5 cpm

of labelled oligonucleotides were mixed with 4 ul of binding buffer (final concentration: 25mM HEPES pH 7.6, 5mM $MgCl_2$ and 34mM KCl), 1μg poly dIdC, 2μg BSA, 2 ul proteinase inhibitor cocktail (20mM DTT, 2mM PMSF) in a total of 10 ul volume and kept on ice. The mix was then added to equal volume of dialysis buffer (25mM Hepes, 1mM EDTA, 40mM KCl, 11.4% glycerine) containing 0, 1 and 3 ug of nuclear extract. The mix was left at room temperature for 15-20 mins before analysis on a non-denaturing 6% polyacrylamide gel.

HBV core protein distribution in vitro

To express the core gene with its homologous promoter, nts 1549-2458 were generated by PCR using C9y & C2y as outer and C8y & C4y as inner primers (Table 11), then cloned into pT7-blue (Novagen, AMS Biotechnology, Oxon, UK). The *PstI-XhoI* fragment was recloned into vector pKLt55 (a generous gift from Dr Walter, Genetics Dept, Glasgow University) whose own promoter had been removed. To study the effects of core protein sequence variability on intracellular distribution without any effects from its homologous promoter, a fragment of nt 1818-2458 containing core gene only, was generated by PCR from the pKLt55 construct using primers C5e and C4h and cloned into vector pRK5 (a generous gift from Prof H Will, Hamburg, Germany) which contains the SV40 origin of replication and the CMV early promoter. Core constructs under the control of the homologous promoter were expressed in HepG2 hepatocyte cells and those under the heterologous promoter were expressed in both Cos7 and HepG2 cells. Briefly, 5ug plasmid DNA was transfected into cells on a 16mm coverslip in 35mm dish by standard calcium phosphate precipitation method. Cells were harvested 48-72 hrs after transfection, fixed with paraformaldehyde, labelled with polyclonal anti-core IgG (Sigma Chemical Company, St Louis, USA) as 1° antibody and anti-rabbit mouse IgG as 2° antibody. Cells were examined using a Nikon Microphot-SA fluorescence microscope.

RESULTS

Fulminant hepatitis B virus constructs have a higher transcription level

A sequence greater than 400 nt containing the NRE, CURS, BCP, Enh II and the 5' end of the core gene from a total of 20 patients was cloned into a luciferase-expressing vector. Eleven of 20 were FHB; 4 of them were HBeAg negative in the first available samples. Two were laboratory standard *adw* (HBV *adw*2 in Genbank; genotype A) and *ayw* (Xxhepav in Genbank; genotype D) subtype controls (both HBeAg producing) and the remaining 7 were CHB controls with well known

clinical characteristics. Table 13 shows the luciferase level correlated with variation in the *cis* acting and pre-core regions. Seven of 11 FHB cases, all with variation in the first two AT rich regions of the BCP (Figure 7) showed substantially higher luciferase activity compared to control *adw* which we considered as background or normal. Six of these 7 cases also had A_{1896} and one with G_{1896} sequence had an A insertion at 1838 and was therefore an HBeAg negative strain. Two of 11 cases (FHBV-8 and FHBV-9) had an intermediate level of luciferase level compared to control *adw*. For case FHBV-8, 2 clones were tested, one with A_{1896} and the other with G_{1896}, but both had variant BCP regions. Both clones showed the same level of luciferase activity, but this was not consistent (higher standard deviation). Case FHBV-9 was G_{1896} and invariant in the BCP, but had a single variant in the CURS. The last 2 of the 11 FHB cases (FHBV-12 and FHBV-16) had normal luciferase activity, and contained G_{1896} with almost identical variation in the CURS and BCP. Only one of these variants was in the third AT rich region and both sequences were invariant in the first two AT rich regions. Six of 7 control CHB cases showed normal luciferase production comparable to *adw* control. Three of 7 CHB controls had HBeAg-producing sequences (G_{1896}), the remainder were A_{1896}.

Turning to specific variants, of those in the BCP, T_{1762} (in three) and A_{1764} (in five) were the most common in CHB (5 of 7 cases). These variants also were common in FHB associated with high luciferase expression but always were accompanied by T_{1766} and/or A_{1768}. This indicates that T_{1762} and A_{1764} alone are not sufficient to affect transcription in our system. However, T_{1762} (without A_{1764}) with one other unique variant (A_{1826}) appeared to be crucial in case FHBV-1. The only CHB control case which had intermediate luciferase activity (I-40), was HBeAg positive and contained a deletion in the BCP from nt1754-1762 (Figure 7). The functional effect of combinations of non-unique variants was further seen in the linkage of variants at nts 1727 and 1740. G_{1727} and T_{1740} together were observed 4 times, but only in the group of 7 high luciferase-producing FHB sequences. The control *ayw* subtype had higher luciferase activity compared to *adw* and had considerable differences in the BCP/enhancer II complex, particularly at positions 1678, 1727, 1740 and 1773 (Figure 7). Con-*ayw* had A_{1727} and C_{1740}; a number of FHB and control CHB cases also contained A_{1727}, but always with T_{1740}. This is a remarkable parallel with our finding of the 4 FHB cases with G_{1727} and T_{1740} . T_{1773} was found in 8/11 of the FHB cases with high or intermediate luciferase activity. Three FHB cases had T_{1678}, 2 of them had high and 1 intermediate luciferase activity; con-*ayw*, the control with intermediate activity was the only control sequence to have T_{1678}. In summary, although the BCP is generally variable, some unique variants, but more importantly, combinations of variants ("motifs"), were associated with high or intermediate transcriptional activity, nearly always in FHB cases.

Luciferase activity correlates with disease progression

There was a correlation between high luciferase expression, rapidity of disease progression, and seroconversion to anti-HBe. All 7 FHB cases with high luciferase expressing sequences had rapid disease progression regardless of their clinical outcomes (Tables 1 and 4). In contrast, FHB cases with intermediate or normal luciferase expression had slow disease progression. There was a correaltion between high luciferase expression and HBeAg status in FHB cases. Six of 7 FHB cases with high luciferase expression were HBeAg negative on admission and seroconverted to anti-HBe, other 4 FHB cases with intermediate and normal luciferase expression were HBeAg positive on admission and 1 of them seroconverted to anti-HBe. In contrast, Four of 7 HBeAg negative CHB cases had normal luciferase expression.

BCP and enhancer I sequences from FHB have different nuclear factor binding patterns to non-FHB controls

To investigate the effect of BCP variation on binding of transcription factors derived from nuclear extracts, complementary oligonucleotides spanning nts 1748-1783 from 6 FHB cases with variability in the BCP and from *adw* subtype as a non-FHB control were synthesised. As can be seen from Figure 7, the chosen oligonucleotides included most of the variants previously identified as linked to FHB. Figure 8 gives the results of nuclear factor binding and Figure 10 compares the sequence to cartoons of the banding patterns. The non-FHB sequence (OL-*adw*) shows 3 clear complexes (Figure 8a), whereas all FHB sequences except one (FHBV-1), which had only one mutation (T_{1762}), bound poorly, or not at all, with complexes II and III. Case FHBV-1 (Figure 8c) which showed a similar binding pattern compared to non-FHB control (OL-*adw*), had two bands in complex-II. In order to identify liver specific complexes, nuclear extracts from HeLa cells were made, and the binding assay repeated using oligonucleotides from 3 FHB and 1 control non-FHB cases. From Figure 9b, it is clear that complexes II and III are not hepatocyte specific. The BCP variant oligonucleotides showed a similar pattern of binding to both nuclear extracts (Figure 9b, lane 3, 4 and 5). A competitive experiment using unlabelled oligonucleotides as a cold competitor showed the specificity of the interactions (Figure 8.d).

A number of FHB sequences had variants in Enh-I, particularly $T_{1050}C$, $G_{1249}C$ and T_{1250}. Four pairs of oligonucleotides with or without Enh-I variants were synthesised (Table 12) and bound to nuclear extracts of HuH7 cells. Figure 11 shows that variant G_{1050} (oligonucleotide em-1) does not have any effect on the nuclear factor binding pattern, but variants T_{1249} and C_{1250} (oligonucleotide em-

2), in contrast to G_{1249} and T_{1250} (oligonucleotide ew-2), do not give rise to complex 3.

Finally, a nuclear factor binding assay was performed using an oligonucleotide with an insertion of A at 1838 (Table 12) as detected in two patients (CHBV-1 and FHBV-15). No difference was observed in the binding pattern (data not shown).

Expression and distribution of core protein was not different in FHBV.

To investigate the pattern of core protein and its intracellular distribution under the control of the homologous promoter, an area spanning nt 1549-2458 from 13 patients (8 FHB or contacts of FHB, 4 CHB and 1 AHB) was cloned into vector pKLt55. Core proteins were expressed in HepG2 cells and labelled with polyclonal anti-core rabbit IgG. The influence of core protein sequence on intracellular distribution was addressed using core nt 1818-2458 from 11 FHB, 4 CHB and 1 AHB cases in vector pRK5 under control of the CMV early promoter. The core proteins were expressed in both COS7 and HepG2 cells. HBcAg distribution was observed in both cytoplasm and nucleus and there was no obvious difference in the level of HBcAg expressed in FHB and control cases. A similar level of expression was observed using either homologous or heterologous promoters (data not shown).

DISCUSSION

A *cis*-acting transcriptional regulatory element of approximately 100 bp (nt 1743-1849) has been shown to function as the BCP (26). This region also contains the 3' end of the X gene and the 5' end of the encapsidation signal and is sufficient for accurate initiation of both pre core and pregenomic RNAs. A sequence element upstream of the BCP, the CURS, binds to hepatocyte nuclear factors HNF-3, HNF-4, C/EBP or other transcription factors, and stimulates the activity of the BCP (27,28,29). Enh-II overlaps the BCP and has a stimulatory effect on the BCP, as well as the SP-I and SP-II promoters (28,29). Three AT rich regions have been mapped within the BCP region, the first two of which serve as the initiation sites for two longer pre-core, and the third one a shorter pregenomic, mRNAs (30). The two most common variants in these AT rich regions are T_{1762} and A_{1764}, observed in association with both chronic HBV carriers and FHB cases. Initially they were believed to be correlated with HBeAg negative phenotype, perhaps acting by downregulating precore mRNA synthesis (30,31). However, this has not been confirmed by others (32), although there may be an association with lower levels of HBeAg (32).

One hypothesis to explain the massive liver cell injury in FHB is that altered binding of

transcription factors leads to increased virus replication which, in predisposed individuals, induces an exaggerated immune response. We have shown clearly that *cis*-acting regions from four phylogenetically linked clusters of FHB viral sequences which are epidemiologically unrelated have enhanced transcriptional activity *in vitro*. Further, this effect was confined to those FHB-associated sequences containing variants in BCP as well as A_{1896}. The only exception to this rule was CHBV-1, a symptomless male contact implicated in causing FHB in successive wives (4). His sequence also showed high luciferase expression despite G_{1896} but this was accompanied by an A insertion at nt1838 and C_{1862} which lies within the encapsidation signal and might have altered the RNA secondary structure. FHB cases with G_{1896} sequences (clusters 3 and 7) had normal or intermediate luciferase levels. Two of these cases with normal transcription activity had variants in the BCP, one of which was in the third AT rich region (at nt 1794); its effect on transcription is thus unknown, but is unlikely to be important. It is illuminating that A_{1896} sequences from chronic carriers not associated with FHB cases had normal transcriptional activity whilst A_{1896} containing contacts of FHB cases had levels similar to A_{1896}-FHB cases. A_{1896} containing chronic carrier controls also had variants in the BCP, commonly T_{1762} and A_{1764}. But in FHB cases these two variants were accompanied by T_{1766}, A_{1768} or both. While this manuscript was in preparation, these latter two variants were found based on sequence generated from a single patient, to be associated with an elevated rate of replication (33). It is clear that the two most common variants in the BCP (T_{1762} and A_{1764}) do not contribute to the level of transcription unless they occur in combination with other variants. This substantiates the conclusions from the phylogenetic analysis that there are multiple sequence motifs in FHB- associated cases which have a common functional outcome and that A_{1896} itself is not, *per se*, the major factor but is a marker for other variation within the *cis* acting region of the genomes associated with it. Clearly, this helps to explain why there are large numbers of persons infected with A_{1896} strains but very few cases of FHB. As FHB as a clinical entity is rarely, if ever, transmitted, it must also be true that this increased transcriptional activity must be suppressed or otherwise offset in chronic carriers who have it, as it was found that core promoter variation in chronic patients has no effect on transcription (34).

The second finding of this work is that, in most cases with increased transcription, binding of one or more nuclear factors is impaired. Oligonucleotides were synthesised which encompass the regions in BCP which bind several transcriptional regulatory proteins including a liver specific factor C/EBP (29). A recent study found that T_{1762} and A_{1764} inhibition of binding of nuclear factors (35) is liver specific, as nuclear extracts from HeLa cells led to fewer bands. In our study, the missing bands were

not liver specific, as nuclear extract from HeLa cells showed the same pattern of binding. Figure 11, which schematically correlates the variation in the BCP with nuclear factor binding patterns, indicates that particular nuclear protein binding patterns are associated with BCP variants. The oligonucleotide containing only one variant (FHBV-1) showed a similar pattern of binding to the non-FHB control, indicating that T_{1762} alone is unlikely to inhibit binding of nuclear factors. However, the oligonucleotide derived from case FHBV-15, which contained one unique variant (C_{1752}) and one variant out of its usual genotype context (A_{1757}) showed binding inhibition. Oligonucleotides from other FHBV cases with variants in the BCP showed a similar pattern of inhibition. This indicates that a number of combinations can lead to lack of binding to transcription factors to a similar functional outcome. All of these variant cases are associated with high or intermediate luciferase activity; we therefore conclude that these two complexes have an inhibitory effect on transcription.

Thirdly, we could not distinguish any differences in HBcAg distribution in liver-derived cells in culture from FHB compared to sequences from chronic carriers. HBcAg was seen in both nucleus and cytoplasm. The amount of HBcAg production was qualitatively similar whether homologous or heterologous promoter systems were employed. In parallel studies (Dornan et al, submitted) in chronic cases, sequential samples showed shifts in distribution of HBcAg from nucleus to cytoplasm. Clearly, if this is of relevance to the pathogenesis of chronic hepatitis, a different mechanism is operating in fulminant hepatitis.

Interestingly, there was a correlation between clinical parameters and transcriptional activity. High transcriptional activitieswere found in "rapid FHB" and their symptomless contacts. Rapid FHB is characterized by undetectable viral antigens with rapid seroconversion to anti-HBe and decline into coma within two weeks of first symptoms and similarly rapid spontaneous recovery. Both symptomless contacts studied here were implicated in rapidly progressive FHB in two successive wives each (4). Liver cell necrosis and ensuing rapid clinical deterioration is explained by triggering a massive immune response which, ultimately, favours clearance of virus. Similarly, early, complete cessation of virus replication should favour liver regeneration and explain the rapid clinical recovery and, ultimately, good prognosis associated with this subgroup of patients with FHB. Clearly, high levels of viraemia are not invariable with high luciferase activities because serum HBV DNA levels typically were low in such contacts and undetectable in these FHB cases except within the first week and only by nested PCR. Also, massive liver injury is not inevitable with high transcriptional activity as both contacts had symptomless mild chronic hepatitis. In contrast, those patients with "slow FHB" typically show a more protracted clinical and serological course, over several weeks, and have lower

transcriptional activities than "rapid FHB". Early complete cessation of virus replication is lacking; seroconversion may be delayed as HBV DNA levels remain detectable for several weeks from first symptoms.

FIGURES 7 to 11

Figure 7: Sequences from nt 1549 to 1974 were cloned into the vector pBL. Here the sequence of CURS, BCP and Enh-II is only shown. Patient numbers were followed as for previous study in Section I. CHBV-1 and -2 are contacts of FHB cases. All I-numbers denote chronic carrier controls. BCP=basal core promoter CURS=core upstream regulatory sequence V=insertion X=deletion.

Figure 8: Nuclear extracts were prepared from HuH7 cells. 0.1 and 3 ug of nuclear extracts were mixed with 30,000 cpm ^{32}p labelled oligonucleotides(oligos) and run on 6% polyacrylamide nondenaturing gel. Figure 8A nuclear factors binding pattern of oligos from adw and CHBV-2. 3.B and 3.C nuclear factor binding pattern of 5 more variant oligos. £.D 30,000 cpm ^{32}p labelled oligonucleotide from FHBV-14 was mixed with 1x, 5x and 25x molar excess of unlabelled oligonucleotide as cold competitor (lanes 3, 4 and 5), added to 3 ug nuclear extract and resolved on 6% polyacrylamide gel, lane 1=no nuclear extract, lane 2= no cold competitor.

Figure 9: Nuclear extracts were made from HuH7 cells and HeLa cells. 0.1 and 3 ug of nuclear extracts were mixed with 30,000 cpm ^{32}p labelled oligonucleotide and resolved on 6% polyacrylamide non denaturing gel. Figure 9a shows binding of oligonucleotide derived from con-adw (OL-adw). CHBV-2, FHBV-14 and FHBV-4 with nuclear extracts from HuH7. Figure 9b shows same oligonucleotides binding with nuclear extract made from HeLa cells.

Figure 10: Cartoon representation of nuclear factor binding assay showing luciferase values and variants in the BCP; and

Figure 11: Oligonucleotides were made from nomal C_{1050}(OL ew-1), variant G_{1050} (OL em-1), normal C_{1249}, T_{1250} (OL ew-2) and variant T_{1249}, C_{1250} (OL em-2). Oligonucleotides were mixed with 0.1 and 3 ug of nuclear extracts from HuH7 cells and run on 6% polyacrylamide non-denaturing gel.

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Table I

Cluster number Sequence number & name	Genes sequences available	Genotype	Age/Sex	HbeAg	Anti-HBe	Disease type & course (rapid/slow)**	Outcome**	Country of origin of infection & comments	Reference
CLUSTERED AND RELATED SEQUENCES									
1									
1.FHBV1	C,X	D	25/F	-	-	FHBV-Rapid	Died@	UK	$
2.FHBV2	C,X	D	23/F	-	+	FHBV-Rapid	Survived	UK	$
3.FHBV3	C,X	D	17/F	-	+	FHBV-Rapid	Survived	UK	$
4.FHBV4	C,X	D	18/F	-	+	FHBV-Rapid	Survived	UK	$
5.FHBV5	C,X	D	19/M	+	-	FHBV-Rapid	Survived	UK	$
6.FHBV6	C,X	D	23/F	-	-	FHBV-Rapid	Survived	UK	$
7.AHBV1	C,X	D	NA	+	-	AHBV-NA	Survived	UK contact of FHBV6	$
8.FHBV7	C,X	D	21/F	-	-	FHBV-Slow	Survived+@	Italy	$
2									
9.FHBV8	C,X	A	25/F	-	+	FHBV-Rapid	Died	UK	$
10.CHBV1	C,X	A	35/M	-	+	CHBV-Rapid	Died	UK,fatally infected two successive wives	6,$
3									
11.FHBV9	C,X	A	23/M	+	-	FHBV-Slow	Died	UK	$
12.FHBV10	C,X	A	30/F	+	-	FHBV-Slow	Died	UK	$
13.*FHBV11	C	A	45/F	+	-	FHBV-Rapid	Died	Nepal	$

14.HBVP3CSX	C,X	D	F	-	NA	FHBV-NA	Survived[±]	Greece	21,24
15.HBVP2CSX	C,X	D	M	NA	NA	FHBV-NA	Died	Greece	24
16.*HBVP1PC	C	D	F	-		CHBV-NA	Survived[±]	Greece, contact of HBVP3CSX	21
17.*HBVP2PC	C	D	M	-	+	CHBV-NA	Survived[±]	Greece, contact of HBVP1PC	21
5									
18.FHBV12	C,X	D	20/F	+	-	FHBV-Slow	Died[β]	Spain	$
19.FHBV13	C,X	D	25/M	-	-	FHBV-Slow	Died[β]	Swiss	$
6									
20.*FHBV14	C,X	D	29/F	-	+	FHBV-Rapid	Died[@]	Pakistan	$
21.CHBV2	C,X	D	35/M	-	+	CHBV-Rapid	Died	UK,fatally infected two successive wives	6,$
22.HPBMUT	C,X	D	NA	-	+	FHBV-Rapid	Died	Israel,one of 5 fatally infected in an outbreak attributable to a single source	5,8
23.*HPBC5HK02	C,X	C	40/F	-	+	CHBV-NA	Died	Japanese, contact of one case	4,25
24.*HPBETNC	C,X	C	infant	-	-	CHBV-NA	Died	Japanese, contact of two cases	7,26
25.HBVP4PCXX	C,X	D/A[§]	M	-	NA	FHBV-NA	Died	Greece	21
26.HBVP5PCXX	C,X	D/A[§]	F	-	+	CHBV-NA	Died	Greece, contact of HBVP4PCCXX	21
INDIVIDUAL UNLINKED CASES									
27.HBVP4CSX	C,X	B	M	-	NA	FHBV-NA	Survived	Chinese	24
28.FHBV15	C	D	27/M	-	+	FHBV-Slow	Survived[±]	USA	$
29.FHBV16	C,X	A	30/M	+	-	FHBV-Slow	Died	Cameroon	$
30. HPBC4HST2	C,X	C	57/M	-	+	FHBV-Slow	died	Japanese	25

Table 2.

Nucleotide Sequence*

Region	
Full sequence	**0.024**
1373-1631	0.906
(CURS)1631-1742	0.678
(BCP)1742-1838	**0.009**

Amino Acid Sequence

Full sequence	**0.050**
1-86	0.255
(CURS)87-123	0.636
(BCP)124-154	**0.042**

Nucleotide Sequence*

Region	FHBV	G_{1896}FHBV	A_{1896}FHBV	A_{1896}non-FHBV
Full sequence (1901-2458)	**0.045**	**0.022**	**0.009**	**0.018**
Region 1 (1963-2020)	0.154	0.787	0.076	0.715
CD4 epitope (2050-2107)	0.610	0.361	0.944	**0.036**
Anti-HBc/e1 (2122-2161)	0.919	0.281	0.141	**0.036**
Anti-HBc/e2 (2200-2251)	0.724	0.281	**0.014**	0.281
Anti-HBc/e3 (2290-2305)	0.787	1.000	**0.022**	0.181
Antigenic regions	0.108	0.052	**0.009**	**0.014**
Non-antigenic regions	**0.039**	**0.043**	**0.013**	0.051

Amino Acid Sequence

Region	FHBV	G_{1896}FHBV	A_{1896}FHBV	A_{1896}non-FHBV
Full sequence	0.108	0.151	**0.013**	**0.013**
Antigenic regions	0.162	0.295	**0.009**	**0.014**
Non-antigenic regions	0.235	0.052	**0.024**	0.183

Table 4.

	EnhancerI				NRE		BCP/Enhancer II*					Pre-C	Aberrant Cysteines				aberrant Methionines			
Nucleotide variant	G_{1050}	T_{1115}	T_{1249}	C_{1250}	1633±	1634±	T_{1762}	A_{1764}	T_{1766}	A_{1768}	1773±	A_{1896}	1390±	T_{1449}	T_{1587}	1605±	A_{1386}	G_{1637}	G_{1754}	A_{1794}
Translation§							M130	I131	Y132			stop	6±	26	72	78±	5±	88	127	141
FHBV Prevalence	10/26	2/26	10/26	14/26	10/26	13/26	6/26	7/26	4/26	2/26	3/26	16/30	2/26	11/26	1/26	1/26	3/26	9/26	5/26	1/26
Non-FHBV Prevalence	4/52	1/52	3/52	3/52	3/88	6/88	16/88	15/88	3/88	3/88	0/88	43/161	2/88	9/88	0/88	1/88	1/88	2/88	0/88	0/88

CLUSTERED AND RELATED SEQUENCES

1																				
1. FHBV1	+	-	+	+	+	-	+	-	-	-	-	+	-	+	-	-	-	+	-	-
2.FHBV2	+	-	+	+	+	+	-	-	-	-	-	+	-	+	+	+	-	+	-	-
3.FHBV3	+	-	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-
4.FHBV4	+	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-	+	+	-
5.FHBV5	+	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+	+	-
6.FHBV6	+	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-
7.AHBV1	+	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+	+	-
8.FHBV7	+	-	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+	-	-
2																				
9.FHBV8	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-
10.CHBV1**	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
3																				
11.FHBV9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.FHBV10	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-

13.FHBV11	NA	NA	NA	NA	NA	NA	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	-	-				
4																								
14.HBVP3CSX	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-				
15.HBVP2CSX	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
16.HBVP1PC	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	NA	NA	NA	NA	NA	NA	NA	NA				
17.HBVP2PC	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	NA	NA	NA	NA	NA	NA	NA	NA				
5																								
18.FHBV12	+	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-				
19.FHBV13	+	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-				
6																								
20.FHBV14	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-				
21.CHBV2	-	-	-	+	-	-	+	+	+	+	+	+	-	-	-	-	+	-	-	-				
22.HPBMUT	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	-	-	-	-	-				
23.HPBC5HK02	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-				
24.HPBETNC	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	+	-	-	-				
25.HBVP4PCXX	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	+	-	-				
26.HBVPCXX	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	+	-	-				
INDIVIDUAL UNLINKED CASES																								
27.HBVP4CSX	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-				
28.FHBV15**	NA	NA	NA	NA	NA	NA	-	-	-	-	-	+	NA	NA	NA	NA	NA	NA	NA	NA				
29.FHBV16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
30.HPBC4HST2	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-				

TABLE 5

	Enh-I	NRE 1633/1634	Enh-II/CURS/BCP
FHB cases in this study			
FHBV-1	G_{1050}, T_{1249}, C_{1250}	+/-	T_{1762} $\underline{A}_{1826}$,
FHBV-2	G_{1050}, T_{1249}, C_{1250}	+/+	-
FHBV-3	G_{1050}, T_{1249}, C_{1250}	+/-	$\underline{T}_{1821}$
FHBV-4	G_{1050}, T_{1249}, C_{1250}	+/+	$\underline{G}_{1754}$
FHBV-5	G_{1050}, T_{1249}, C_{1250}	+/+	$\underline{G}_{1754}$
FHBV-6	G_{1050}, T_{1249}, C_{1250}	+/+	$\underline{G}_{1754}$
FHBV-7	G_{1050}, T_{1249}, C_{1250}	+/+	-
FHBV-8	-	-/-	T_{1762} A_{1764}
FHBV-9	-	-/-	C_{1740} C_{1773}
*FHBV-10	-	-/-	-
*FHBV-11	n/a	n/a	-
FHBV12	G_{1050}, T_{1249}, C_{1250}	+/+	$\underline{A}_{1653}$, $\underline{C}_{1703}$, G_{1754}
FHBV13	G_{1050}, T_{1249}, C_{1250}	+/+	C_{1773}
FHBV14	G_{1050}, T_{1249}, C_{1250}	-/-	T_{1764} G_{1766}
FHBV15	n/a	n/a	$\underline{C}_{1752\ 1838}AC_{1839}$
FHBV16	-	-/-	$\underline{T}_{1703}$ $\underline{T}_{1809}$ $\underline{T}_{1812}$
CHBV-1	-	-/-	$A_{1764\ 1838}A_{1839}$
CHBV-2	G_{1050}, C_{1250}	-/-	T_{1762} A_{1764} T_{1766} A_{1768}
AHBV-1	G_{1050}, T_{1249}, C_{1250}	+/+	$\underline{G}_{1754}$
FHB cases from Genbank			
HBVP3CSX	-	-/+	-
HBVP2CSX	-	-/+	-
HBVP1PC	n/a	n/a	n/a
HBVP2PC	n/a	n/a	n/a
HPBMUT	-	-/-	T_{1762}, A_{1764}, T_{1766}, A_{1768}
HPBC5HK02	-	-/-	A_{1764}, T_{1768}
HBVP4PCXX	-	-/-	T_{1762}, A_{1764}
HBVP5PCXX	-	-/+	C_{1773}
HBVP4CSX	-	-/+	C_{1773}
HPBC4HST2	-	-/+	T_{1762}, A_{1764}, T_{1768}

TABLE 5-2

X protein Cys/Met	X protein Unique	A_{1896}	Clinical factors	Comments/ Motif (Table 6 refes)
C26 M88 M130	L151	+	Pregnant	Motifs- all
C26 C72 M88	F75	+		Motifs- 1, 2, 3, 6, 7, 8
C26 M88		-	-	Motifs- 6, 7, 8
C26 M88 M127	F6	+	-	Motifs- 1, 2, 3
C26 M88 M127	T68	-	-	Motifs- 6, 7, 8
C2, M127		-	-	Motifs- 6, 7, 8
C26 M88	-	-	Pregnant	Motifs- 6, 7, 8
C6 M130	Y95 D126	-	-	Motifs- 4, 5
-	-	-	-	1 unusual variant plus variant outside genotype context
-	-	-	-	-
n/a	n/a	-	-	-
C26 M127	-	-	HDV	Motifs 4, 5, 6,7 plus HDV coinfection
C26	N88	-	HDV	Motifs 6,7 plus HDV coinfection
-	A146, S147	+	Pregnant	Motif 1,3 plus pregnant
n/a	n/a	+	-	Unique variant in BCP plus insertion
-	A146, S147	-	-	Unique variants in BCP and X protein
M130 M141	S13, L150	-	-	Motif- 5 plus insertion
M5 M130	T127	+	-	Motif- 2,3,5
C26 M88 M127	-	-	-	Motifs- 6,7,8
-	?	+	-	A_{1896} plus variants in NRE
-	?	-	-	Variants in NRE
n/a	?	+	-	A_{1896}

TABLE 5-3

n/a	?	+	-	A_{1896}
C26, M130	?	+	-	Motifs-1, 2, 4, 5
M5,	?	+	-	Motifs- 2,5
M5, M130	?	+	-	Motifs- 2,5
M88	?	+	-	Motifs- 2, 5 plus C_{1773}
M88	?	+	-	Motifs-2, 5 plus C_{1773}
M130	?	+	-	A_{1896} plus variants in BCP

TABLE 6

Position	Typical variant at that position for a given genotype	Incidence of deviation from these associations	
		FHBV	Non-FHBV
Nucleotide			
1633	G_{1633} (A, F), A_{1633} (B, C, D)	11/26	7/88
1634	G_{1634} (A, B, C, F), A_{1634} (D)	13/26	6/88
1773 *	T_{1773} (A), C_{1773} (B, C, D, E, F)	3/26	0/88
Amino Acid			
6**	Tyrosine/Phenylalanine (A), Cysteine (B, C, D, F)	2/26	2/88
78**	Cysteine (A), Arginine/Serine (B, C, D, F)	1/26	1/88

TABLE 7-1

MOTIFS *	1	2	3	4	5	6	7	8	A1896 Sub-Total	Total
FHBV Prevalence	4/26	8/26	12/26	11/26	14/26	4/26	8/26	8/26	11/16	21/26
Non-FHBV Prevalence	1/88	0/88	4/88	2/88	1/88	0/88	0/88	1/88	1/88	6/88
1										
1. FHBV1	+	+	+	+	+	+	+	+		
2. FHBV2	-	-	+	+	+	+	+	+		
3. FHBV3	-	-	+	+	+	-	-	-		
4. FHBV4	-	-	+	+	+	+	+	+		
5. FHBV5	-	-	+	+	+	-	-	-		
6. FHBV6	-	-	+	+	+	-	-	-		
7. AHBV1	+	-	+	+	+	-	-	-		
8. FHBV7	-	+	+	+	+	-	-	-		

TABLE 7-2

2								
9. FHBV8	+	-	-	-	-	-	-	-
10. CHBV1	-	+	-	-	-	-	-	-
3								
11. FHBV9	-	-	-	-	-	-	-	-
12. FHBV10	-	-	-	-	-	-	-	-
4								
14. HBVP3CSX	-	-	-	-	+	-	-	+
15. HBVP2CSX	-	-	-	-	+	-	-	-
5								
18. FHBV12	-	-	+	+	+	-	-	-
19. FHBV13	-	-	+	-	+	-	-	-
6								
20. FHBV14	-	-	-	-	-	-	-	+
21. CHBV2	-	+	-	-	-	-	+	+
22. HPBMUT	+	-	-	-	-	+	-	-

TABLE 7-3

23. IIPBCSHKO2	-	+	-	-	-	-	+	-
24. IIPBETNC	-	+	-	-	-	-	+	-
25. IIBVP4PCXX	-	+	+	+	+	-	+	+
26. IIBVP5PCXX	-	+	+	+	+	-	+	+
INDIVIDUAL CASES								
27. IIBVP4CSX	-	-	-	-	-	-	-	-
29. IIIBV16	-	-	-	-	-	-	-	-
30. IIPBC4IIST2	-	-	-	-	-	-	-	-

table 8

Number	Variants				Incidence	
	A +	B +	C +	D	FHBV	Non-FHBV
1.	A1896	T1762	A1764		4/26	1/88
2	T1762	A1764	T1766		3/26	1/88
3.	T1762	A1764	T1766	T1768	3/26	1/88.
4.	A1896	A1386			3/26	0/88
5.	A1896	G1637			5/26	0/88
6.	A1896	T1449			4/26	0/88
7.	A1896	G1050			3/26	1/52
8.	A1896	T1249			4/26	0/52
9.	A1896	C1250			7/26	0/52
10.	T1762	T1449			2/26	0/88
11.	T1762	A1386			1/26	0/88
12.	T1762	G1637			1/26	0/88
13.	T1764	1390 *			1/26	0/88
14.	T1764	T1449			1/26	0/88
15.	T1764	A1386			3/26	0/88
16.	T1764	A1794			1/26	0/88
17.	T1766	T1449			1/26	0/88
18.	T1766	A1386			2/26	0/88
19.	T1768	T1449			1/26	0/88
20.	T1768	A1386			1/26	0/88
21.	1773 *	T1449			1/26	0/88
22.	1773 *	A1386			3/26	0/88
23.	C1050	1633 *			10/26	1/52
24.	C1050	1634 *			8/26	0/52
25.	T1249	1633 *			9/26	1/52
26.	T1249	1634 *			7/26	0/52
27.	C1250	1633 *			10/26	0/52

TABLE 8-2

28.	C1250	1634 *	10/26	0/52	
23.	G1637	1633 *	7/26	2/88	
23.	G1637	1634 *	7/26	2/88	
24.	G1754	1633 *	5/26	0/88	
24.	G1754	1634 *	5/26	1/88	

TABLE 9

Sequence	Variant																				
	G1050	T1115	T1249	C1250	A1386	1390	T1449	T1587	1605	1633	1634	G1637	G1754	T1762	A1764	T1766	A1768	1773	A1796	A1896
HBVORFS	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.
XXHEPAV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HBVGEN1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.
XXHEPAV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HPBHBVAA	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	.	.	X	.	X
HBVAYWMCG	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X
HBVDNA	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AHBV1	X	.	X	X	.	.	X	.	.	X	X	X	X	.	.	.	.	X	.	.
FHBV1	X	.	X	X	.	.	X	.	.	X	.	X	.	X	.	.	.	X	.	X
FHBV2	X	.	X	X	.	.	X	X	X	X	X	X	.	.	.	.	.	X	.	X
FHBV3	X	.	.	X	.	.	X	.	.	X	.	X	.	.	.	.	.	X	.	.
FHBV4	X	.	X	X	.	.	X	.	.	X	X	X	X	.	.	.	.	X	.	X
FHBV5	X	.	X	X	.	X	X	.	.	X	X	X	X	.	.	.	.	X	.	.
FHBV6	X	.	X	X	.	.	X	.	.	X	X	.	X	.	.	.	.	X	.	.
FHBV7	X	.	X	X	.	.	X	.	.	X	X	X	.	.	.	.	.	X	.	.
FHBV12	X	.	X	X	.	-	-	.	.	X	X	.	X	.	.	.	.	X	.	.
FHBV13	X	.	X	X	.	.	X	.	.	X	X	.	.	.	.	.	.	X	.	.
HBVP3CSX	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	X
HBVP2CSX	.	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.
CHBV2	.	.	.	X	X	.	.	.	.	.	.	.	.	X	X	X	X	.	.	X
HPBMUT	.	.	.	.	.	.	X	.	.	.	.	.	.	X	X	X	X	.	.	X
FHBV14	.	.	X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X

TABLE 10-1

Name	Gender/age	Country of origin	Days of illness before sample	HBsAg*	HBeAg	Anti-HBe	HBV DNA (dot-blot)	Transmission route	Clinical features
FHB cases and contacts									
FHBV-1	F/25	UK	8	N/D	-ve	-ve	N/D	?	Pregnant. Died
FHBV-4	F/18	UK	6	1: 64	-ve	+ve	-ve	Sexual	Survived
FHBV-5	M/19	UK	5	1:6,400	+ve	-ve	-ve	IV use	Survived
FHBV-8	F/25	UK	8	1:64	-ve	+ve	N/D	Sexual	Died
FHBV-9	M/23	UK	14	1:3,200	+ve	-ve	+ve	Sexual	Died
FHBV-12	F/20	UK	14	1;800	+ve	-ve	+ve	IV use	Died
FHBV-14	F/30	Pakistan	4	1:6,400	-ve	+ve	N/D	?	Pregnant. Died
FHBV-15	M/27	USA	9	N/D	-ve	+ve	-ve	Sexual	Transplanted
FHBV-16	M/30	Cameroon	19	1:6,400	+ve	-ve	+ve	Percutaneous	Died

WO 98/45421

table 10-2

CHBV-1	M/50	UK	N/A	1:800	-ve	+ve	N/D	N/A	Contact carrier
CHBV-2	M/40	Pakistan	N/A	1:800	-ve	+ve	N/D	N/A	Contact carrier
Chronic carrier controls									
I-40	21/F	Italy	N/A	++	+ve	-ve	+ve	N/A	Chronic carrier
I-59	58/F	Italy	N/A	+ve	+ve	-ve	+ve	N/A	Chronic carrier
I-69	31/M	Italy	N/A	+ve	+ve	-ve	+ve	N/A	Chronic carrier
I-89	27/M	Italy	N/A	+ve	-ve	+ve	+ve	N/A	Chronic carrier
I-95	49/M	Italy	N/A	+ve	-ve	+ve	+ve	N/A	Chronic carrier
I-105	33/M	Italy	N/A	+ve	-ve	+ve	+ve	N/A	Chronic carrier
I-177		Italy	N/A	+ve	-ve	+ve	+ve	N/A	Chronic carrier

TABLE 11

Name	Sequences	Restriction site	Position
C8m	5'-TCGACGGATCCGTCTGTGCCTTCTCATCTGC	*Bam H1*	sense
BC3	5'-GCATTCTGCAGGAAAGAAGTCAGAAGGCAA	*Pst 1*	anti-sense
C9y	5'-CGAGCTCGAGACCACGGGGCGCACCTCTCTTTAC	*Xho 1*	sense, outer
C8y	5'-CGAGCTCGAGGTCTGTGCCTTCTCATCTGCC	*Xho 1*	sense, inner
C2y	5'-GACCTGCAGCCCCCA/CGTAAAGTTTCCC/GACCTT	*Pst 1*	anti sense, outer
C4y	5'-GACCTGCAGCCTTATGAGTCCAAGGG/AATA	*Pst 1*	anti sense, inner
C5e	5'-AGTCGAATTCCA/CCCTCTGCCTAACATCTC	*Eco R1*	sense
C4h	5'-GGACAGAAGCTTCCTTATGAGTCCAAGGG/ATA	*Hin dIII*	anti sense

TABLE 12

OL-*adw*	1748	GGAGATTAGGTTAAAGGTCTTTGTATTAGGAGGCTG	1783
OL-FHBV-1		----------T----------C----------	
OL-FHBV-4		-----C---A----T-A-T-A----------	
OL-FHBV-5		-----C---A----T-A-T-A----------	
OL-FHBV-14		---------A------T-G----------	
OL-FHBV-15		----C----A----------C----------	
OL-CHBV-2		-----C---A----T-A-T-A--C----------	
OL-EW1	1031	CACAATGTGGTTATCCTGCGTTAATGCCCTTGTATG	1066
OL-EM1		----------T--------T-------	
OL-EW2	1231	CGCATGCGTGGAACCTTTGTGGCTCCTCTGCCGATC	1266
OL-EM2		----------TC----------	
OL-lt.wt	1783	GTAGGCATAAATTGGTCTGCGCACCAGCACCATGC	
OL-lt.in		----------A--------	
		AACTTTTTCACCTCTGCCTAA-TCAC 1842	
		----------A----	

TABLE 13

Phylogenetic Cluster	Patients and controls	Luciferase (x 10^6)	Standard deviation ±	Pre-core	Variants in the Enh-II/ CURS/BCP	Variants in the Enh-II/ CURS/ BCP (found in that particular clone)	Disease progression
1	FHBV-1	11.51 (H)	3.57	A_{1896}	T_{1762} A_{1836}	T_{1762} A_{1826}	Rapid
	FHBV-4	8.81 (H)	1.77	A_{1896} A_{1899}	G_{1754}	C_{1753} T_{1762} A_{1764} T_{1766} A_{1768} T_{1810}	Rapid
	FHBV-5	10.25 (H)	2.29	A_{1896} A_{1899}	G_{1754}	C_{1753} T_{1762} A_{1764} T_{1766} A_{1768} T_{1810}	Rapid
2	FHBV-8.1	2.76 (I)	1.64	A_{1896} A_{1899}	T_{1762} A_{1764}	$\underline{T}_{1678}$ T_{1764} G_{1766} T_{1845}	Slow
	FHBV-8.5	3.65 (I)	1.16	G_{1896}	N/A	$\underline{C}_{1740}$ C_{1753} T_{1762}	Slow
	CHBV-1	10.70 (H)	0.82	G_{1896}	$A_{1764\ 1838}A_{1839}$	$\underline{C}_{1740\ 1838}$ A_{1839}	Rapid
3	FHBV-9	4.06 (I)	0.58	G_{1896}	C_{1740} C_{1773}	$\underline{C}_{1740}$ C_{1773}	Slow
5	FHBV-12	0.60 (N)	0.30	G_{1896}	A_{1653} C_{1703} G_{1754}	T_{1703} C_{1794} T_{1809} T_{1812} C_{1821}	Slow
6	FHBV-14	11.39 (H)	4.71	A_{1896} A_{1899}	T_{1764} G_{1766}	$\underline{T}_{1678}$ T_{1764} G_{1766} A_{1834} T_{1845}	Rapid
	CHBV-2	9.68 (H)	2.46	A_{1896} A_{1899}	T_{1762} A_{1764} T_{1766} A_{1768}	C_{1753} T_{1762} A_{1764} T_{1766} A_{1768} C_{1771} T_{1810}	Rapid
Individual cases	FHBV-15	7.98 (H)	1.12	A_{1896}	$C_{1752\ 1838}AC_{1839}$	$\underline{T}_{1678}$ $C_{1752\ 1838}AC_{1839}$	Rapid
	FHBV-16	1.13 (N)	0.22	G_{1896}	T_{1703} T_{1809} T_{1812}	T_{1703} A_{1794} T_{1809} T_{1812} C_{1821}	Slow

TABLE 13-2

Controls	I-40	4.18 (I)	1.61	G_{1896}	N/A	Δ1754-1762 A_{1732} T_{1733} A_{1764} A_{1768}	N/A
	I-59	0.77 (N)	0.36	G_{1896}	N/A	C_{1719}	N/A
	I-69	0.55 (N)	0.29	G_{1896}	N/A	-	N/A
	I-89	0.78 (N)	0.30	A_{1896} A_{1899}	N/A	T_{1762} A_{1764}	N/A
	I-95	0.56 (N)	0.28	A_{1896} A_{1899}	N/A	C_{1719} T_{1762} A_{1764} T_{1811}	N/A
	I-105	1.25 (N)	0.79	A_{1896} A_{1899}	N/A	C_{1753} T_{1762} A_{1764}	N/A
	I-177	0.62 (N)	0.39	A_{1896}	N/A	C_{1721} A_{1764}	N/A
	adw	1.5 (N)	0.84	G_{1896}		C_{1740}	N/A
	ayw	4.21 (I)	1.74	G_{1896}		T_{1678} C_{1740}	N/A

CLAIMS

1. Hepatitis B virus polynucleotide for use in evaluation of a hepatitis B disease state which comprises at least two of the following:

(i) a mutation in the Enhancer I region;

(ii) a mutation in the Negative Regulatory Element region;

(iii) a mutation in the Enhancer II/Core Upstream Regulatory Sequence/Basal Core Promoter region; and

(iv) a mutation which leads to an X peptide amino acid change to provide a cysteine or methionine residue;

the mutation being a variation from the normal nucleotide at that position in a respective one of HBV genotypes A to F.

2. Hepatitis B virus polynucleotide for use in detection of fulminant hepatitis B viral infection which comprises at least two of the following:

(i) a mutation at one or more of the following positions 1050, 1249 and 1250;

(ii) a mutation at one or more of the following positions 1633 and 1634;

(iii) a mutation at one or more of the following positions 1653, 1754, 1762, 1764, 1766, 1768, 1809, 1821, 1826 and 1838/9 insertion;

(iv) a mutation which leads to an X-peptide change to provide cysteine or methionine at one or more of amino acid positions 26, 72, 88, 127 and 130;

the mutations being variations from the normal nucleotide at that postion in a respective one of HBV genotypes A to F.

3. Hepatitis B virus polynucleotide for use in detection of fulminant hepatitis B viral infection which comprises at least two of the following:

(i) a mutation which provides one or more of the following nucleotides at the following positions. G(1050), T(1249) and C(1250);

(ii) a mutation which provides one or more of the following nucleotides at the following positions.

A, T or C (1633) for genotype A or F

T, G or C (1633) for genotype B, C or D

A, T or C (1634) for genotype A, B, C or F

T, G or C (1634) for genotype D;

(iii) a mutation which provides one or more of the following nucleotides at the following positions.

A(1653), G(1754), T(1762), A(1764), T(1766), A(1768), T(1809), T(1821), A(1826), and AC (insertion between 1838 and 1839);

(iv) a mutation which leads to an X-peptide change to provide one of the following amino acids at the following amino acid positions.

C(26), M(88), M(130), M(127), M(141), M(5) and C(72).

4. Hepatitis B Virus polynucleotide for use in the detection of fulminant hepatitis B viral infection according to any of motifs 1 to 5 of Table 7 herein.

5. Hepatitis B virus polynucleotide for use in the detection of fulminant hepatitis B viral infection having at least two nucleotides in the positions according to any of numbers 1 to 31 in Table 8 herein.

6. Hepatitis B virus X-peptide having one of the following amino acids at the following amino acid positions:

C(26), M(88), M(130), M(127), M(141), M(5) and C(72).

7. Polynucleotide probe having a sequence complementary to that of any of claims 1 to 5.

8. Polynucleotide probe having a sequence complementary to a polynucleotide sequence coding for an HBV X-peptide of claim 6.

9. A test kit for detection of an HBV disease state which comprises polynucleotide fragment probes capable of hybridising under appropriate stringency conditions to any two of (i), (ii), (iii) and (iv) of claims 1, 2 or 3.

10. A test kit according to claim 7 comprising probes capable of hybridising to all four of (i) to (iv).

11. Antibody to any of the X peptides of claim 6.

12. An immunoassay for the detection of fulminant HBV which comprises an antibody to any of the X peptides of claim 6.

13. A test method for determining binding interactions between host or viral proteins and HBV polynucleotides which comprises;

- taking a polynucleotide fragment according to any of claims 1 to 6;
- applying said host or viral protein thereto; and
- determining the degree of binding between the protein and the polynucleotide fragment.

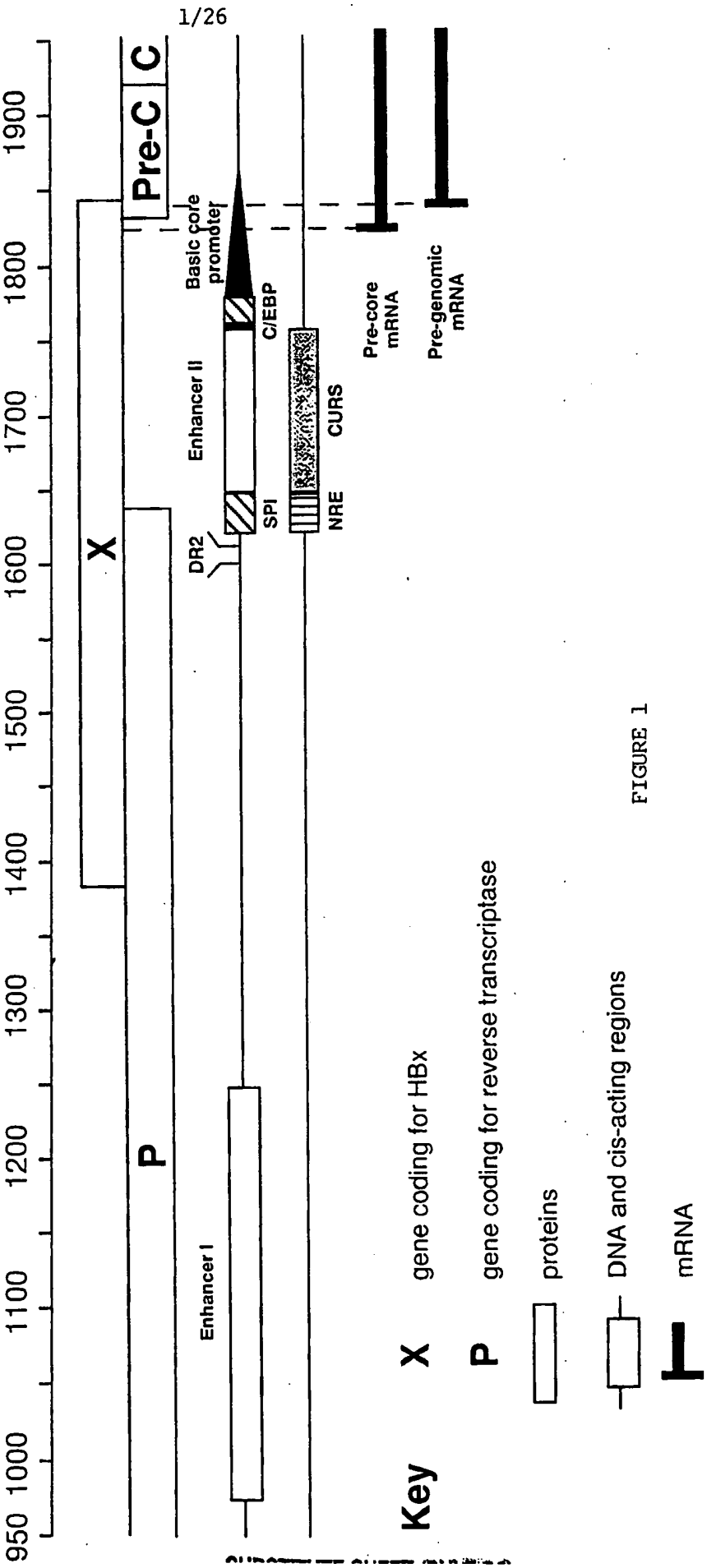

950
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
X
P
Pre-C
C
Enhancer I
Enhancer II
Basic core promoter
C/EBP
DR2
SPI
NRE
CURS
Pre-core mRNA
Pre-genomic mRNA
Key
X gene coding for HBx
P gene coding for reverse transcriptase
proteins
DNA and cis-acting regions
mRNA

FIGURE 1

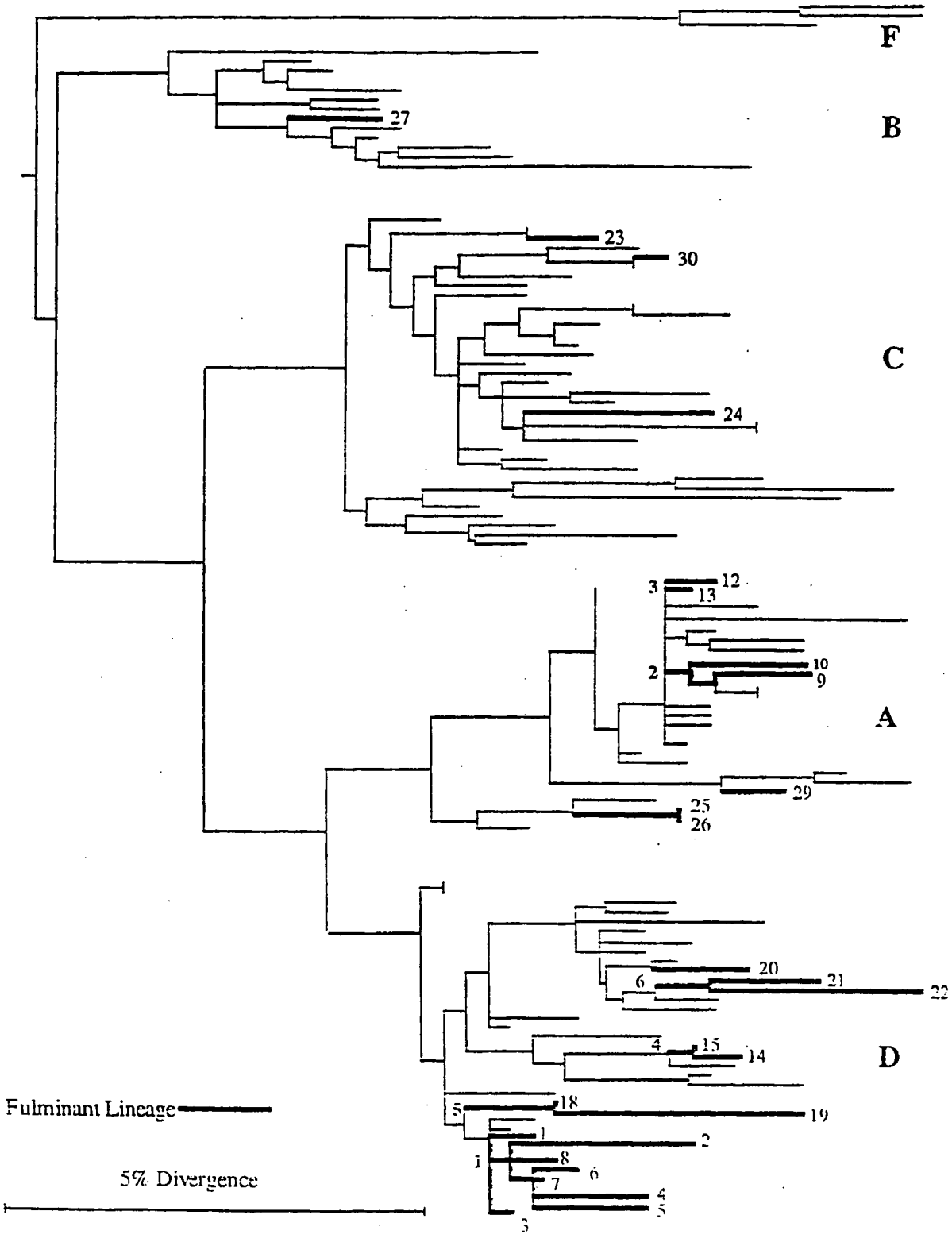

F
B
27
C
23
30
24
A
3
12
13
2
10
9
29
25
26
D
20
6
21
22
4
15
14
5
18
19
1
2
1
8
6
7
4
5
3
Fulminant Lineage
5% Divergence

FIGURE 2


F
B
27
24
23
C
30
29
11
3
12
13
A
9
2
10
22
6
21
20
25
26
28
15
16
4
17
14
D
5
18
19
8
2
3
5
4
1
1
7
6
Fulminant Lineage
5% Divergence

FIGURE 3

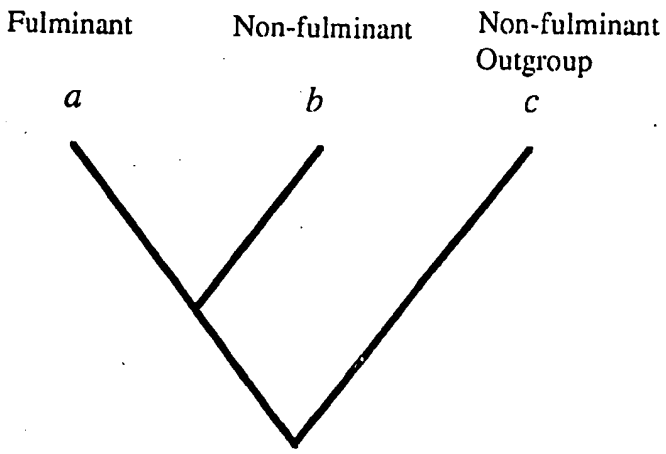


FIGURE 4

```
Name        Sequences
----        ---------------------------------------------------------------
            1001
HBVORFS     TGGGTCTTTT GGGTTTTGCT GCCCCTTTTA CACAATGTGG TTATCCTGCT TTAATGCCCT
XXHEPAV     .......... .......... .......... .......... .......... ........T.
HBVGEN1     .......... .......... .......... .......... .......... ....A...T.
HBVAYWMCG   .....T.A.. .......... .......... .......... .........C ..G.....T.
HBVDNA      .......... .......... ..T..A.... .......... .........G ........T.
XXHEPAV     .......... .......... .......... .......... .......... ........T.
HPBHBVAA    .......... ...A...... ..T....... .......... .......... ..........
AHBV1       .......... .......... .....C.... .......... .........G ..........
FHBV1       .......... ...C...... .......... .......... .........G ..........
FHBV2       .......... A......... .......... .......... .........G .........C
FHBV3       .......... .......... .....A.... .......... .........G ..........
FHBV4       .......... .......... .......... .......... .........G ..........
FHBV5       .......... ...G...... .....C.... .......... .........G ..........
FHBV6       .......... .......... .....C.... .......... .........G ..........
FHBV7       .......... .......... .......... .......... .........G ..........
FHBV12      .........G ...C....T. ..G..C.... .........A .........G ........T.
FHBV13      .......... .......... .......... .......... .........G ..........
HBVP3CSX    .......... .......... .......... .......... .......... ..........
HBVP2CSX    .......... .......... .......... .......... .......... ..........
CHBV2       .......... ...G...... .......... ........AA .......... ....A...T.
HPBMUT      .A........ .......... .......... .T........ .......... ....A.....
FHBV14      .......... .......... .......... .......... .......... ..G.......
            1061
HBVORFS     TGTATGCCTG TATTCAATCT AAACAGGCTT TCACTTTCTC GCCAACTTAC AAGGCCTTTC
XXHEPAV     .......A.. ......G..G ..G....... .T........ .......... ..........
HBVGEN1     .......... ......G... ..G....... .......... .......... ..........
HBVAYWMCG   .......A.. .......... ..G....... .......... .......... ..........
HBVDNA      .......... ...A...G.. .......... .......... .......... ..........
XXHEPAV     .......A.. ......G..G ..G....... .T........ .......... ..........
HPBHBVAA    .......A.. .......... ..G....... .......... .......... ..........
AHBV1       .......... .......... ..G....... .......... .......... ..........
FHBV1       .......A.. .......... ..G....... .......... .......... ..........
FHBV2       .......A.. .......... ..G....... .......... .......... ..........
FHBV3       .......A.. .......... ..G....... ..T....... .......... ..........
FHBV4       .......A.. .......... ..G....... .......... .......... ..........
FHBV5       .......A.. .......... ..G....... .......... .......... ..........
FHBV6       .......... .......... ..G....... .......... .......... ..........
FHBV7       G......A.. .......... ..G....... .......... .......... ..........
FHBV12      .......A.. .......... ..G....... .......... .......... ..........
FHBV13      .......A.. .......... ..G....... .......... .......... ..........
HBVP3CSX    .......... .......G.. .....A.... .......... .......... ....T.....
HBVP2CSX    .......... .......G.. ......A... .......... .......... ....T.....
CHBV2       C..C...AA. ...A...... .......... .......... .......... ..........
HPBMUT      .......A.. .......... .......... .......... .......... ..........
FHBV14      .......A.. .......... ..G....... .......... .......... ..........
            1121
HBVORFS     TGTGTAAACA ATACCTGAAC CTTTACCCCG TTGCTAGGCA ACGGCCAGGT CTGTGCCAAG
XXHEPAV     .......... .......... .......... ....CC.... .......... ..........
HBVGEN1     .......... ...T...... .......... ....CC.... .......... ..........
HBVAYWMCG   .......... .......... .......... ....CC.... .......... ..........
HBVDNA      .AA....... G...A..... .......... .....C.... ......T... ..........
XXHEPAV     .......... .......... .......... ...CC.... .......... ..........
```

FIGURE 5-1

```
HPBHBVAA  ..........  ..........  ..........  ....CC....  ...C......  ..........
AHBV1     ......C...  ..........  ..........  C...CC....  ..........  ..........
FHBV1     ..........  ..........  ..........  ....CC....  ..........  ..........
FHBV2     ..........  ..........  ..........  ....CC....  ..........  ..........
FHBV3     ..........  ..........  ..........  ....CC....  ..........  ..........
FHBV4     ..........  ..........  .......G..  ....CCA...  ..........  ..........
FHBV5     ..........  ..........  ..........  ....CC....  ..........  ..........
FHBV6     ..........  ..........  ..........  C...CC....  ..........  ..........
FHBV7     ..........  ..........  ..........  ....CC....  ..........  ..........
FHBV12    ..........  ..........  ..........  ....CC....  ..........  ..........
FHBV13    ..........  ..........  ..........  ....CC....  ..........  ..........
HBVP3CSX  ..........  ..........  ..........  ..........  ..........  ..........
HBVP2CSX  ..........  ..........  ..........  ..........  ..........  ..........
CHBV2     ..........  ..........  .......T..  ....CC....  ..........  ..........
HPBMUT    ..........  .......C..  ..........  ....CC....  ..........  ..........
FHBV14    ..........  ..........  ..........  ....CC....  ......W...  ..........

          1181
HBVORFS   TGTTTGCTGA CGCAACCCCC ACTGGCTGGG GCTTGGTCAT GGGCCATCAG CGCATGCGTG
XXHEPAV   ..........  ..........  ..........  ..........  ..........  ..........
HBVGEN1   ..........  ..........  ..........  ..........  ..........  ..........
HBVAYWMCG ..........  ..........  ..........  ..........  ..........  ..........
HBVDNA    ..........  ..........  ..........  ......CATA  ..........  ..........
XXHEPAV   ..........  ..........  ..........  ..........  ..........  ..........
HPBHBVAA  ..........  ..........  ..........  ..........  ..........  ..........
AHBV1     ..........  ..........  ..........  ..........  ..........  ..........
FHBV1     ..........  ..........  ..........  ..........  ..........  ..A...A...
FHBV2     .....CG...  ..........  ..........  .......A..  ..........  ..........
FHBV3     ..........  ..........  ..........  ..........  ..........  ..........
FHBV4     ..........  ..........  ..........  ..........  ..........  ..........
FHBV5     ..........  ..........  ..........  ..........  ..........  ..........
FHBV6     ..........  ..........  ..........  ..........  ..........  ..........
FHBV7     .....T....  ..........  ..........  ..........  ..........  .........T
FHBV12    ...C.TG...  ..........  ..........  ..........  ..........  ..........
FHBV13    .....C....  ..........  ..........  ..........  ..........  ..........
HBVP3CSX  ..........  ..........  ..........  ..........  ..........  ..........
HBVP2CSX  ..........  ..........  ..........  ..........  ..........  ..........
CHBV2     ..........  ..........  ..........  ..........  ..........  ..........
HPBMUT    ..........  ..........  ..........  ..........  ..........  ...G......
FHBV14    ..........  ..........  ..........  ..........  ..........  ..........

          1241
HBVORFS   GAACCTTTCT GGCTCCTCTG CCGATCCATA CTGCGGAACT CCTAGCCGCT TGTTTTGCTC
XXHEPAV   ..........  .....G....  ..........  ..........  ..........  ..........
HBVGEN1   ..........  ..........  ..........  ..........  ..........  ..C.......
HBVAYWMCG .........G  ..........  ..........  ..........  ..........  ..........
HBVDNA    ........GA  ..........  ..........  ..........  ..........  ..........
XXHEPAV   ..........  .....G....  ..........  ..........  ..........  ..........
HPBHBVAA  .........A  ..........  ..........  ..........  ..........  ..........
AHBV1     ........TC  ..........  ..........  ..........  ..........  ..........
FHBV1     ........TC  ..........  .T.....T..  ..........  ..........  ..........
FHBV2     ........TC  ..........  ..........  ..........  ..........  ..........
FHBV3     ........GC  ..........  ..........  ..........  ..........  ..........
FHBV4     ........TC  ..........  ..........  ..........  ..........  ..........
FHBV5     ........TC  ..........  ..........  ..........  ..........  ..........
FHBV6     ........TC  ..........  ..........  ..........  ..........  ..........
FHBV7     ........TC  ..........  ..........  ..........  ..........  ..........
FHBV12    ........TC  ..........  ..........  ..........  ..........  ..........
FHBV13    ........TC  ..........  ..........  ..........  ..........  ..........
HBVP3CSX  ..........  ..........  ..........  ..........  ..........  ..........
```

FIGURE 5-2

```
HBVP2CSX    .......... .......... .......... .......... .......... ..........
CHBV2       .........C .......... .......... .......... .......... ..........
HPBMUT      .......... .......... .......... .......... .......... ..........
FHBV14      .........TC .......... .......... .......... .........C ..........

            1301
HBVORFS     GCAGCAGGTC TGGAGCAAAC ATTCTCGGGA CGGATAACTC TGTTGTTCTC TCCCGCAAAT
XXHEPAV     .......... .......... .......... .......... .......... ..........
HBVGEN1     .......... ...G...... .......... .......... .......... ..........
HBVAYWMCG   .......... .......... .......... .........T ......C..A ..........
HBVDNA      .......... .......... ...A...... .T........ ......C..A ..G..G....
XXHEPAV     .......... .......... .......... .......... .......... ..........
HPBHBVAA    .....C.... .......... .......... .......... .......... ..........
AHBV1       .......... .......... ...A...... .T........ ......C... ..........
FHBV1       .......... .......... ...A...... .T........ ......C... ..........
FHBV2       .......... .......... ...A...... .T........ ......C... ..........
FHBV3       .......... .......... ...A...... .T........ ......C... ..........
FHBV4       .......... .......... ...A...... .T........ ......C... ..........
FHBV5       .......... .......... ...A...... .T........ ......C... .........N
FHBV6       .......... .......... ...A...... .T........ ......C... ..........
FHBV7       .......... .......... ...A...... .T........ ......C... ..........
FHBV12      .......... .......... ...A...... .T........ ......C... ..........
FHBV13      .......... .......... ...A...... .T........ ......C... ..........
HBVP3CSX    .......... .......... .......... .......... .......... ..........
HBVP2CSX    .......... .......... .......... .T........ .......... ..........
CHBV2       .......... .......... ..C....... ....C..... C......... ..........
HPBMUT      .......... .......... ...A...... .......... .......... ..........
FHBV14      .......... .......... .......... .......T.. .......... ..........

            1361
HBVORFS     ATACATCGTT TCCATGGCTG CTAGGCTGTG CTGCCAACTG GATCCTGCGC GGGACGTCCT
XXHEPAV     .........A .......... .......... .......... .......... ..........
HBVGEN1     .......... .......... .......... .......... .......... ..........
HBVAYWMCG   .......... .......... .......... .......... .......A.. ..........
HBVDNA      .......... .......... .....T...A .......... ......T... ..........
XXHEPAV     .........A .......... .......... .......... .......... ..........
HPBHBVAA    ....G..... .......... .......... .......... .......... ..........
AHBV1       .........A .......... .......... .......... .......... ..........
FHBV1       .......A.A .......... .......... .......... .......... ..........
FHBV2       ......T..A .......... .......... .......... .......... .......T.C
FHBV3       .........A C......... .......... .......... .......... ..........
FHBV4       .........A .......... .......... .......... .......... ..........
FHBV5       N..TC....A .......... .........T .......... .......... ..........
FHBV6       .........A ...G...... .......... .......... .......... ..........
FHBV7       .........A .......... .......... .......... .......... .T........
FHBV12      .........A .......... .........- ---------- ---------- ----------
FHBV13      .......A.A .......... .......... .......... .......... ..........
HBVP3CSX    .......... .......... .......... .......... .......... ..........
HBVP2CSX    .......... .......... .......... .......... .......... ..........
CHBV2       ......... . .......... .....A.... .......... .......... ..........
HPBMUT      .......... .......... .......... .......... .........A ..........
FHBV14      .......... .......... .......... .......... .......... ..........

            1421
HBVORFS     TTGTTTACGT CCCGTCGGCG CTGAATCCCG CGGACGACCC TTCTCGGGGC CGCTTGGGGA
XXHEPAV     .......... .......... .......... .......... .........T ........AC
HBVGEN1     .......... .......... .......... .......... .......... ........AC
HBVAYWMCG   .C........ .......... ........A. .......... .......... ........AC
HBVDNA      .......... .......... .......... .......... C......... ........AC
XXHEPAV     .......... .......... .......... .......... .........T ........AC
HPBHBVAA    .......... .......... .......... .......... .......... ........AC
```

FIGURE 5-3

```
AHBV1          .......... .......... .......T. .......... .........T .........AC
FHBV1          .......... .......... .......T. .......... .........T .........AC
FHBV2          .......... .......... .......T. .......... .........T .........AC
FHBV3          .......... .......... .......T. .......... .........T .........AC
FHBV4          .......... .......... .......T. .......... C......... T.......AC
FHBV5          .......... T......... .......T. .......... .........T .........AC
FHBV6          .......... .......... .......T. .......... .........T .........AC
FHBV7          .......... .......... .......T. .......... .........T .........AC
FHBV12         ---------- ---------- ---------- ---------- ---------- ----------
FHBV13         .......... .......... .......T. .......... .........T .........AC
HBVP3CSX       .......... .......... .......... .......... .......... ..........
HBVP2CSX       .......... .......... .......... .......... .......... ..........
CHBV2          .......... .......... .......... .......... .......... .........AC
HPBMUT         ....C..... .......... .......T. .......... .........T .........AC
FHBV14         .......... .......... .......... .......... .......... .........AC

               1481
HBVORFS        TCTTTCGTCC CCTTCTCCGT CTGCCGTTCC GTCCGACCAC GGGGCGCACC TCTCTTTACG
XXHEPAV        ...C...... .......... .......T. .A........ .......... ..........
HBVGEN1        ...C...... .......... .......T. .A........ .......... ..........
HBVAYWMCG      ...C...... .......... .......T. .......... .......... ..........
HBVDNA         ...C...... .......... .......... AG........ .......... ..........
XXHEPAV        ...C...... .......... .......T. .A........ .......... ..........
HPBHBVAA       ...C...... .......... .......T. .A........ .......... ..........
AHBV1          ...C...... .......... .......... .A........ .......... ..........
FHBV1          ...C...... .......... .......... .A........ .......... ..........
FHBV2          ...C...... .......... .......... .A........ .......... ..........
FHBV3          ...C...... .......... .......... .A........ .......... ..........
FHBV4          ...C...... .......... .GT....... .A........ .......... ..........
FHBV5          ...C...... .......... .......... .A........ .......... ..........
FHBV6          ...C...... .......... .......... .A........ .......... ..........
FHBV7          ...C...... .......... .......... .A........ .......... ..........
FHBV12         ---------- ---------- .......... ..A....... .......... .....C....
FHBV13         ...C...... .......... .......... .A........ .......... ..........
HBVP3CSX       .......... .......... .......T. .......... .......... ..........
HBVP2CSX       .......... .......... .......T. .......... .......... ..........
CHBV2          ...C...... .......... .......T. .A........ .......... ..........
HPBMUT         ...C...... .......... .......T. .A........ .......... ..........
FHBV14         ...C...... .......... .......T. .A........ .......... ..........

               1541
HBVORFS        CGGACTCCCC GTCTGTGCCT TCTCATCTGC CGGTCCGTGT GCACTTCGCT TCACCTCTGC
XXHEPAV        .......... .......... .......... ...A...... .......... ..........
HBVGEN1        .......... .......... .......... ...A...... .......... ..........
HBVAYWMCG      .......... .......... .......... ...A...... .......... ..........
HBVDNA         ...T...A.. .......... .......... .......... .......... ..........
XXHEPAV        .......... .......... .......... ...A...... .......... ..........
HPBHBVAA       .......... .......... .......... ...A...... .......... ..........
AHBV1          .......... .......... .......... ...A...... .......... ..........
FHBV1          .......... .......... .......... ...A...... .......... ..........
FHBV2          .......... .......... .......... ...A...... .......T... .T..T.T...
FHBV3          .......... .......... .......... ...A...... .........G. ..........
FHBV4          .......... .......... .......... ...A...... .......... ..........
FHBV5          .......... .......... .......... ....A..... .......... .T........
FHBV6          .......... .......... .......... ...A...... .......... ..........
FHBV7          .......... .......... .......... ...A...... .......... ..........
FHBV12         .......... .......... .......... ...A...... .......... ..........
FHBV13         .......... .......... .......... ...A...... .......... ..........
HBVP3CSX       .......... .......... .......... ...A...... .......... ..........
HBVP2CSX       .......... .......... .......... ...A...... .......... ..........
```

FIGURE 5-4

```
CHBV2       .......... .......... .......... ....A..... .......... ..........
HPBMUT      .......... .......... .......... ...A...... .......... ..........
FHBV14      .......... .......... .......... ...A...... .......... ..........
            1601
HBVORFS     ACGTCGCATG GAGACCACCG TGAACGCC-- -CACCACTTC TTGCCCAAGG TCTTACATAA
XXHEPAV     .......... .......... ....A..... ...A.CA... .......... ..........
HBVGEN1     .......... .......... .......... ......A... .......... ..........
HBVAYWMCG   .......... .......... .......... ......A... .......... ..........
HBVDNA      .......... .......... .......... ...T..AAGT C......... ..........
XXHEPAV     .......... .......... ....A..... ...A.CA... .......... ..........
HPBHBVAA    .......... .......... .......... ......A... .......... ..........
AHBV1       .......... .......... .......... .....GGA.G .......... ..........
FHBV1       .......... .......... .......... .....GAA.G .......... ..........
FHBV2       ....T..... .......... .......... .....GGA.G .......... ..........
FHBV3       .......... .......... .......... .....GAA.G .......... ..........
FHBV4       .......... .......... .......... .....GGA:G .......... ..........
FHBV5       .......... .......... .......... .....GGA.G .......... ..........
FHBV6       .......... .......... .......... .....GGA.. .......... ..........
FHBV7       .......... .......... .......... .....GGA.G .......... ..........
FHBV12      .......... .......... .......... .....GGAA. C......... .....AC...
FHBV13      .......... .......... .......... .....GGAA. C......... ..........
HBVP3CSX    .......... .......... .......... .......... .......... ..........
HBVP2CSX    .......... .......... .......... .......... .......... ..........
CHBV2       .......... .......... .......... ......A... .......... ..........
HPBMUT      .......... .......... ........GC C..T.TA... .......... .....T....
FHBV14      .......... .......... .......... ......A... .......... ..........
            1658
HBVORFS     GAGGACTCTT GGACTCTCAG CAATGTCAAC GACCGACCTT GAGGCATACT TCAAAGACTG
XXHEPAV     .......... .........T. T......... .......... .......... ..........
HBVGEN1     .......... .........T. T......... .......... .......... ..........
HBVAYWMCG   .......... .......... .......... .......... .......... ..........
HBVDNA      .......... ......C... .......... .......... .....C.... ..........
XXHEPAV     .......... .........T. T......... .......... .......... ..........
HPBHBVAA    .......... .........T. T......... .......... .......... ..........
AHBV1       .........  .........T. .......... .......... .......... ..........
FHBV1       .......... .........T. .......... .......... .......... ..........
FHBV2       .......... .........T. .......... .......... .......... ..........
FHBV3       .......... .........T. .......... .......... .......... ..........
FHBV4       .......... .........T. .......... .......... .......... ..........
FHBV5       .......... .........T. .......... .......... .......... ..........
FHBV6       .......... .........T. .......... .......... .......... ..........
FHBV7       .......... .........T. .C........ .......... .......... ..........
FHBV12      .......... .........GT .......... .......... .......... ..........
FHBV13      .......... .........TA .......... .......... .......... ..........
HBVP3CSX    .......... .........T. T......... .......... .......... ..........
HBVP2CSX    .......... .........T. .......... .......... .......... ..........
CHBV2       .......... .........T. .......... .......... .......... ..........
HPBMUT      .......... .........T. T......... .......... .......... ..........
FHBV14      .......... .........T. T......... .......... .......... ..........
            1718
HBVORFS     TTTGTTTAAG GACTGGGAGG AGTTGGGGGA GGAGATTAGA TTAAAGGTCT TTGTACTAGG
XXHEPAV     .........A .......... .......... .......... .......... .....T....
HBVGEN1     .........A .......... .......... ....G..... .......... ..........
HBVAYWMCG   .........A ..G....... .......... .......... ......T.G. .....T....
HBVDNA      .G........ .......... ..C....... .........G .......... .....T....
XXHEPAV     .........A .......... .......... .......... .......... .....T....
HPBHBVAA    .......... .......... ...C...... .......... ....T.A... ..........
AHBV1       .........A .......... .......... ......G..G .......... ..........
```

FIGURE 5-5

```
FHBV1        .........A .......... .......... .........G ....T..... ..........
FHBV2        .........A .......... .......... .......... .......... ..........
FHBV3        .........A .......... .......... .........G .......... ..........
FHBV4        .........A .......... .......... ......G..G .......... ..........
FHBV5        .........A .......... .......... ......G..G .......... ..........
FHBV6        .........A .......... .......... ......G..G .......... ..........
FHBV7        .........A .......... .......... .........G .......... ..........
FHBV12       .........A .......... .......... ......G..G .......... ..........
FHBV13       .........A .......... .......... .........G .......... ..........
HBVP3CSX     .........A .......... ....T..... .......... .......... ..........
HBVP2CSX     .........A .......... .......... .......... .......... ..........
CHBV2        .......... .......... .......... .....C.... ....T.A.T. A....T....
HPBMUT       .........A .......... .......... ...T.A.... ....T.A.T. A....T....
FHBV14       .........A .......... .......... .......... ......T.G. .....T....
             1778
HBVORFS      AGGCTGTAGG CATAAATTGG TCTGCGCACC AGCACCATGC AACTTTTTCA CCTCTGCCTA
XXHEPAV      .......... .......... .......... .......... .......... ..........
HBVGEN1      .......... .......... .......... .......... .......... ..........
HBVAYWMCG    .......... .......... .......... .......... .......... ..........
HBVDNA       .......... .......... .......... .......... .......... ..........
XXHEPAV      .......... .......... .......... .......... .......... ..........
HPBHBVAA     .......... .......... .......... .......... .......... ..........
AHBV1        .......... .......... .......... .......... .......... ..........
FHBV1        .......... .......... .......... .......... ........A. ..........
FHBV2        .......... .......... .......... .......... .......... ..........
FHBV3        .......... .......... .......... .......... .......... ..........
FHBV4        .......... .......... .......... .......... .......... ..........
FHBV5        .......... .......... .......... .......... .......... ..........
FHBV6        .......... .......... .......... .......... .......... ..........
FHBV7        .......... .......... .......... .......... .......... ..........
FHBV12       .......... .......... .......... .......... .......... ..........
FHBV13       .......... .......... .......... .......... .......... ..........
HBVP3CSX     .......... .......... .......... .......... .......... ..........
HBVP2CSX     .......... .......... .......... .......... .......... ..........
CHBV2        .......... .......... .......... .......... .......... ..........
HPBMUT       .......... .......... .......... .......... .......... ..........
FHBV14       .......... .......... .......... .......... .C........ ..........
             1838
HBVORFS      A-TCATCTCT TGTTCATGTC CTACTGTTCA AGCCTCCAAG CTGTGCCTTG GGTGGCTTTG
XXHEPAV      .......... .......... .......... .......... .......... ..........
HBVGEN1      .........A .......... .......... .......... .......... ..........
HBVAYWMCG    .......... .......... .......... .......... .......... .........A
HBVDNA       .......... .......... .C........ .......... .......... ..........
XXHEPAV      .......... .......... .......... .......... .......... ..........
HPBHBVAA     .......... .......... .......... .......... .......... .........A
AHBV1        .......... .......... .......... .......... .......... ..........
FHBV1        .......... .......... .......... .......... .......... .........A
FHBV2        .......... .......... .......... .......... .......... .........A
FHBV3        .......... .......... .......... .......... .......... ..........
FHBV4        .......... .......... .......... .......... .......... .........A
FHBV5        .......... .......... .......... .......... .......... ..........
FHBV6        .......... .......... .......... .......... .......... ..........
FHBV7        .......... .......... .......... .......... .......... ..........
FHBV12       .......... ...A...... .C........ .......... .......... ..........
FHBV13       .......... .......... .......... .......... .......... ..........
HBVP3CSX     .......... .......... .......... .......... .......... .........A
HBVP2CSX     .......... .......... .......... .......... .......... ..........
CHBV2        .......... .......... .......... .......... .......... .........A
```

FIGURE 5-6

```
HPBMUT      .........G ..........  .........  .........  .........  .........A
FHBV14      ........T. ..........  .........  .........  .........  .........A
            1897
HBVORFS     GGGCATGGAC ATTGACCCTT ATAAAGAATT TGGAGCTACT GTGGAGTTAC TCTCATTTTT
XXHEPAV     ..........  .....T....  .........  .........  .........  ....G.....
HBVGEN1     ..........  ..C.......  .........  .........  .........  ....G.....
HBVAYWMCG   ..A.......  .....T....  .........  .......T..  A.......G.  ....G.....
HBVDNA      ..........  ..........  .........  .........  .........  ....G.....
XXHEPAV     ..........  .....T....  .........  .........  .........  ....G.....
HPBHBVAA    ..A.......  .....T....  .........  .........  .........  ....G...C.
AHBV1       ..........  ..C.......  .........  .........  .........  ....G....A
FHBV1       ..........  ..C.......  .........  .........  .........  ....T.....
FHBV2       ..........  ..C.......  .........  .......T..  .........  ....G.....
FHBV3       ..........  ..C.......  .........  .........  .........  ....G.....
FHBV4       ..........  ..C.......  .........  .........  .........  ....G.....
FHBV5       ..........  ..C.......  .........  .........  .........  ....G.....
FHBV6       ..........  ..C.......  .........  .........  .........  ....G.....
FHBV7       ..........  ..C.......  .........  .........  .........  ....G.....
FHBV12      ..........  ..C.......  .........  .........  .........  ....G.....
FHBV13      ..........  ..C.......  .........  .........  .........  ....G.....
HBVP3CSX    ..A.......  .....T....  .........  .........  .........  ....T.....
HBVP2CSX    ..........  ..........  .........  .........  .........  .........
CHBV2       ..A.......  .....T....  .........  ...C.....  .........  ....G.....
HPBMUT      ..A.......  ..C.......  .........  .........  ..C.......  ....G.....
FHBV14      ..A.......  .....T....  .........  .........  .........  ....G.....
            1957
HBVORFS     GCCTTCTGAC TTTTTTCCTT CGGTACGAGA TCTTCTAGAT ACCGCCTCAG CTCTGTATCG
XXHEPAV     ..........  ..C.......  .A.......  ..........  .A.......  ..........
HBVGEN1     ..........  ..C.......  .A.......  ..........  ..........  ....C.....
HBVAYWMCG   ..........  ..C.A.....  .A.......  ..........  ..........  ....A.....
HBVDNA      ..........  ..C.......  .C...A....  ...C.....C  ..........  ..........
XXHEPAV     ..........  ..C.......  .A.......  ..........  .A.......  ..........
HPBHBVAA    ..........  ..C.......  .A.......  ..........  ..........  ....A.....
AHBV1       ..........  ..C.......  ..A.G.....  ..........  .G.......  ..........
FHBV1       ..........  .CC.......  .A.......  ..........  ..........  ..........
FHBV2       ..........  ..C.......  .A.......  ..........  ..........  .G........
FHBV3       ..........  C.C.......  .A.......  ..........  ..........  ..........
FHBV4       ..........  ..C.......  .A.......  ..........  ..........  ..........
FHBV5       ..........  ..C.......  .A.......  ..........  ..........  ..........
FHBV6       ..........  ..C.......  .A.......  ..........  ..........  ..........
FHBV7       ..........  ..C.......  .A.......  ..........  ..........  ..........
FHBV12      ..........  ..C.......  .A.......  ..........  ..........  ..........
FHBV13      ..........  ..C.......  .A.......  ..........  ..........  ...GA....C
HBVP3CSX    ....CA....  ..C.......  ..C.......  ..........  ..........  ..........
HBVP2CSX    ..........  ..C.......  ..........  ..........  ..........  ..........
CHBV2       ..........  ..C......G TC........  ..........  ..........  ....A.....
HPBMUT      ..........  ..C.......  .C.C......  ..........  ..........  ......T...
FHBV14      ....A.....  ..C.......  .C........  ..........  ..........  ....A.....
            2017
HBVORFS     GGATGCCTTA GAGTCTCCTG AGCATTGTTC ACCTCACCAT ACTGCACTCA GGCAAGCAAT
XXHEPAV     ...A......  ..........  ..........  ..........  ..........  ..........
HBVGEN1     ...A......  ..........  ..........  ..........  ..........  ..........
HBVAYWMCG   ...A......  ..........  ........A.  ......T...  ..........  ..........
HBVDNA      A..A......  ..........  .......C..  ..........  ..........  .......C..
XXHEPAV     ...A......  ..........  ..........  ..........  ..........  ..........
HPBHBVAA    ...A......  ..A.......  ..........  ..........  ..........  ..........
AHBV1       ...A......  ..........  ..........  ..........  ..........  ..........
FHBV1       ...A......  ..........  ..........  ..........  ..........  ..........
```

FIGURE 5-7

```
FHBV2          ...A..... .......... .......... .......... .......... ..........
FHBV3          ...A..... .......... ...G...... .......... .......... ..........
FHBV4          A..A..... .......... .......... .......... .......... ..........
FHBV5          ...A..... .......... .......... .......... .......... .......C..
FHBV6          ...A..... .......... .......... .......... .......... ..........
FHBV7          ...A..... .......... .......... .......... .......... ..........
FHBV12         ...A..... .......... .......... .....CA... .......... ..........
FHBV13         ...A..... .......... ..AC...... .......... .......... ..........
HBVP3CSX       .......... .......... .......... .......... .......... ..........
HBVP2CSX       .......... .......... ........A. .......... .......... ..........
CHBV2          .......... ..A....... .......... .......... .......... ..........
HPBMUT         ...A..... .......... .......... .......... .......... ..........
FHBV14         ...A..... .......... .......... .......... .......... ..........
               2077
HBVORFS        TCTTTGCTGG GGGGAACTAA TGACTCTAGC TACCTGGGTG GGTGTTAATT TGGAAGATCC
XXHEPAV        A..G..... .......... .......... .......... ....G..... ..........
HBVGEN1        .......... .......... .......... .......... .......... ..........
HBVAYWMCG      .......... ......T... .......... C......... ....G..... ..C.......
HBVDNA         ...C..... ........G. .......... AT........ ....A..... ..........
XXHEPAV        A..G..... .......... .......... .......... ....G..... ..........
HPBHBVAA       ...C..... .....T.... .A......T. C......... ....G..... ..........
AHBV1          .......... .......... .......... .......... .......... ..........
FHBV1          .......... .......... .......... .......... .......... .........A
FHBV2          .......... .......... .......... .......... .......... ..........
FHBV3          .......... .......... .......... .......... .......... ..........
FHBV4          ...A..... .......... .......... .......... ....A..... ..........
FHBV5          .......... ........C. .......... .......... ....C..... ..........
FHBV6          .......... .......... .......... .......... .......... ..........
FHBV7          .......... .......... .......... .......... .......... ..........
FHBV12         .......... .......... .......... .......... .......... ..........
FHBV13         .......... .......... .......... .......... .......... ..........
HBVP3CSX       A......... .....CT... ...A...... .........A .......... ..C.......
HBVP2CSX       C......... .......... .......... .......... .......... ..C.......
CHBV2          .......... .......... .........G C......... ....G..... ..........
HPBMUT         ...G..... .....TG... ...A...... .......... ....GC.... ..........
FHBV14         ...A..... ......T... .......... C......... ....G..... ..........
               2137
HBVORFS        AGCATCTAGG GACCTAGTAG TCAGTTATGT CAACACTAAT ATGGGCCTAA AGTTCAGGCA
XXHEPAV        .AT...C... .......... .......... .......... .......... .A........
HBVGEN1        .......... ........T. .T........ .......... ......T... ..........
HBVAYWMCG      .A....C... .......... .......... T......... .......... ..........
HBVDNA         ...G..... .......... .......... T......... .......... ..A.......
XXHEPAV        .AT...C... .......... .......... .......... .......... .A........
HPBHBVAA       .A........ .......... .......... T......... .......... ..........
AHBV1          .........A .......... .......... .......... .......... ..........
FHBV1          .........A .......... .......... .......... .......... ..........
FHBV2          .........A ..A.AT.... .......... ......AT.. .......... ..........
FHBV3          .........A .......... .......... .......... .......... ..........
FHBV4          .........A .......... .......... .......... .......... ..........
FHBV5          .........A .......... .......... .......... .......... ..........
FHBV6          .........A .......... .......... .......... .......... ..........
FHBV7          .........A .......... .......... .......... .......... ..........
FHBV12         .........A .......... .......... .......... .......... ..........
FHBV13         .........A .......... .......... .......... .......... ..........
HBVP3CSX       .......... ..A....... ...A...... T......... .......... ..........
HBVP2CSX       .......... .......... .......... T......... .......... ..........
CHBV2          ..T...C... .......... .......... T......C.. .........C. ....T.....
HPBMUT         .A....C... ......A.T. ...A...... .......... ........T. G.........
```

FIGURE 5-8

```
FHBV14      .A....C... .......... .T......C. T.......... .......... ....T.....
           2197
HBVORFS     ACTATTGTGG TTTCACATTT CTTGTCTCAC TTTTGGAAGA GAAACAGTCA TAGAGTATTT
XXHEPAV     .......... .......... .......... .......... ........T. ..........
HBVGEN1     ...T...... .......... .......... .......... .....G.... ..........
HBVAYWMCG   .......... ......G... .......... .......... .........G ..........
HBVDNA      .......... .....T..A. ....C..T.. .......... ..G..T..AC .T..A.....
XXHEPAV     .......... .......... .......... .......... ........T. ..........
HPBHBVAA    .......... .......... .......... .......... .....G.... ..........
AHBV1       ...C...... .......... .......... .......... .....C..T. ..........
FHBV1       ...C...... .......... .......... .......... .....C..T. ..........
FHBV2       ...T...... .......... .......... .......... .....C..T. ..........
FHBV3       ...T...... .......... .......... .......... .....C..T. ..........
FHBV4       ...C...... .......... .......... .......... .....C..TC ..........
FHBV5       ...C...... .......... .......... .......... .....C..T. ..........
FHBV6       ...M...... .....T..A. ....C..Y.. .......... ..G..T..AC .C..A.....
FHBV7       ...C...... .......... .......... .......... .....C..T. ..........
FHBV12      ...C...... .......... .......... .......... ........T. ..........
FHBV13      ...C...... .......... .......... .......... .....C..T. ..........
HBVP3CSX    .......... ......G... .......... .......... .......... ..........
HBVP2CSX    ...C...... .......... .......... .......... .....G..T. ..........
CHBV2       .......... .......... .......... .......... ..T..T..AC AT..T.....
HPBMUT      .......... .....TC... .......... .......... .......... ..........
FHBV14      .......... .......Y.. .......T.T G......... .....G...C .T........
           2257
HBVORFS     GGTGTCTTTC GGAGTGTGGA TTCGCACTCC TCCAGCTTAT AGACCACCAA ATGCCCCTAT
XXHEPAV     .........T .......... .......... .......... .......... ..........
HBVGEN1     .......... .......... .......... .......... .......... ..........
HBVAYWMCG   .........T .......... .......... ..A....... .......... ..........
HBVDNA      ...C...... .......... .......... ......C... .......... ..........
XXHEPAV     .........T .......... .......... .......... .......... ..........
HPBHBVAA    .........T .......... .......... .......... .......... ..........
AHBV1       .......... .......... .......... .......... .......... ..........
FHBV1       .......... .......... .......... .......... .......... ..........
FHBV2       .......... .......... .......... .......... .......... ..........
FHBV3       .......... .......... .......... .......... .......... ..........
FHBV4       .......... .......... .......... ..A....... .......... ..........
FHBV5       .......... .......... .......... .......... .......... ..........
FHBV6       ...C...... .......... .......... .......... .......... ..........
FHBV7       .......... .......... .......... .......... .......... ..........
FHBV12      A......... .......... .......... .......... .......... ..........
FHBV13      .......... .......... .......... .......... .......... ..........
HBVP3CSX    .......... .......... .......... ..A....... .......... ..........
HBVP2CSX    .......... .......... .......... .......... .......... ..........
CHBV2       .........T .......... .......... .......... ......T... ..........
HPBMUT      .......... .......... .......... ..A....... .......... ..........
FHBV14      .......... .......... .......... .......... .......... ..........
           2317
HBVORFS     CTTATCAACA CTTCCGGAAA CTACTGTTGT TAGACGA--- ---CGAGGCA GGTCCCCTAG
XXHEPAV     .......... ........G. .......... .......... .......... ..........
HBVGEN1     .......... ........G. .......... .......... .......... ..........
HBVAYWMCG   .......... ........G. ..TG...... .......... .......... ..........
HBVDNA      .......... .......... .......... .......... .....G.A.. ..........
XXHEPAV     .......... ........G. .......... .......... .......... ..........
HPBHBVAA    .......... ........G. .......... .......... .......... ..........
AHBV1       .C........ .......... .......... .......... .......... ..........
FHBV1       .C........ .......... .......... .......... .......... ..........
FHBV2       .C........ .......... .......... .......... .......... ..........
```

FIGURE 5-9

```
FHBV3       .C..... ..... ..... ..... ..... .....
FHBV4       .C..... ..... ..... ..... ..... .....
FHBV5       .C..... ..... ..... ..... ..... .....
FHBV6       .C..... ..... ..... ..... ..... .....
FHBV7       .C..... ..... ..... ..... ..... .....
FHBV12      .C..... ..... ..... ..... ..... .....
FHBV13      .C..... ..... ..... G..... ..... .....
HBVP3CSX    ..... ..... .....A. ..... ..... ..A....
HBVP2CSX    ..... .....G. ..... .....A.... ..... .....
CHBV2       ..... .....G. ..GG.A..A. .....A.CGG GAC..... .....
HPBMUT      ..... .....G. ..... ..... ..... .....
FHBV14      ..... .....G. .....A. ..... ..... .....
            2371
HBVORFS     AAGAAGAACT CCCTCGCCTC GCAGACGAAG ATCTCAATCG CCGCGTCGCA GAAGATCTCA
XXHEPAV     ..... ..... ..... G..... ..... .....
HBVGEN1     ..... ..... ..... ..... ..... .....
HBVAYWMCG   ..... ..... ..... G..... ..... .....
HBVDNA      ..... ..... ..... G..... ..... .....
XXHEPAV     ..... ..... ..... G..... ..... .....
HPBHBVAA    ..... ..... ..... G..... ..A..... .....A....
AHBV1       ..... ..... ..... G..... ..... .....
FHBV1       ..... ..... ..... G..... ..... .....
FHBV2       ..... ..... ..... G..... ..... .....A.
FHBV3       ..... .....A.... ..... G.....A ..... ..GAG.....
FHBV4       ..... ..... .....G. ---------- ---------- ----------
FHBV5       ..... ......G--- ---------- ---------- ---------- ----------
FHBV6       ..... ..... ..... G..... ..... .....
FHBV7       ..... ..... ..... G..... ..... .....
FHBV12      ..... ..... ..... G..... ..... .....
FHBV13      ..... ..... ..... G..... ..... .....
HBVP3CSX    ..... ..... ..... ..... ..... .....
HBVP2CSX    ..... ..... ..... ..... ..... .....
CHBV2       ..... ..... .....C.. ..... ..... .....
HPBMUT      ..... ..... ..C..... GC..... ..... .....
FHBV14      ..... ..... ..... G..... ..... .....
            2431
HBVORFS     ATCTCGGGAA TCTCAATGTT AGTATTCCTT GGACTCATAA AGTGGGTAAC TTTACGGGGC
XXHEPAV     ..... ..... ..... ..... G.....A... .....
HBVGEN1     ..... ..... ..... ..... G.....A... .....A....
HBVAYWMCG   ..... ..... ..... ..... G.....A... .....
HBVDNA      ..... ..... ..... ..... G.....G... .....T....
XXHEPAV     ..... ..... ..... ..... G.....A... .....
HPBHBVAA    ..... ..... .....C. ..... G.....A... .....
AHBV1       ..... ..... ..... ..... G.....G... .....T..T.
FHBV1       ..... .....----- ---------- ---------- ---------- ----------
FHBV2       ..... .....----- ---------- ---------- ---------- ----------
FHBV3       ..... C..... ..... ..... G.....G... .....T..T.
FHBV4       ---------- ---------- ---------- ---------- ---------- ----------
FHBV5       -------... .....----- ---------- ---------- ---------- ----------
FHBV6       ..... ..... ..... ..... G.....G... .....T..T.
FHBV7       ..... .....----- ---------- ---------- ---------- ----------
FHBV12      ..... .....----- ---------- ---------- ---------- ----------
FHBV13      ..... .....----- ---------- ---------- ---------- ----------
HBVP3CSX    ..... ..... ..... ..... ..... .....
HBVP2CSX    .....T.... ..... ..... ..... G.....A... .....
CHBV2       ..... .....C.... ..... ..... G.....A... .....
HPBMUT      ........G. ..... ..... ..... G.....A... .....
FHBV14      ..... C..... ..... ..... G.....A... .....
```

FIGURE 5-10

```
          2491
HBVORFS   TTTATTCCTC
XXHEPAV   .......T..
HBVGEN1   .......T..
HBVAYWMCG .......T..
HBVDNA    .......T..
XXHEPAV   .......T..
HPBHBVAA  .......T..
AHBV1     .......T..
FHBV1     ----------
FHBV2     ----------
FHBV3     .......T..
FHBV4     ----------
FHBV5     ----------
FHBV6     .......T..
FHBV7     ----------
FHBV12    ----------
FHBV13    ----------
HBVP3CSX  .......T..
HBVP2CSX  .......T..
CHBV2     ..........
HPBMUT    .......T..
FHBV14    .......T..
```

FIGURE 5-11

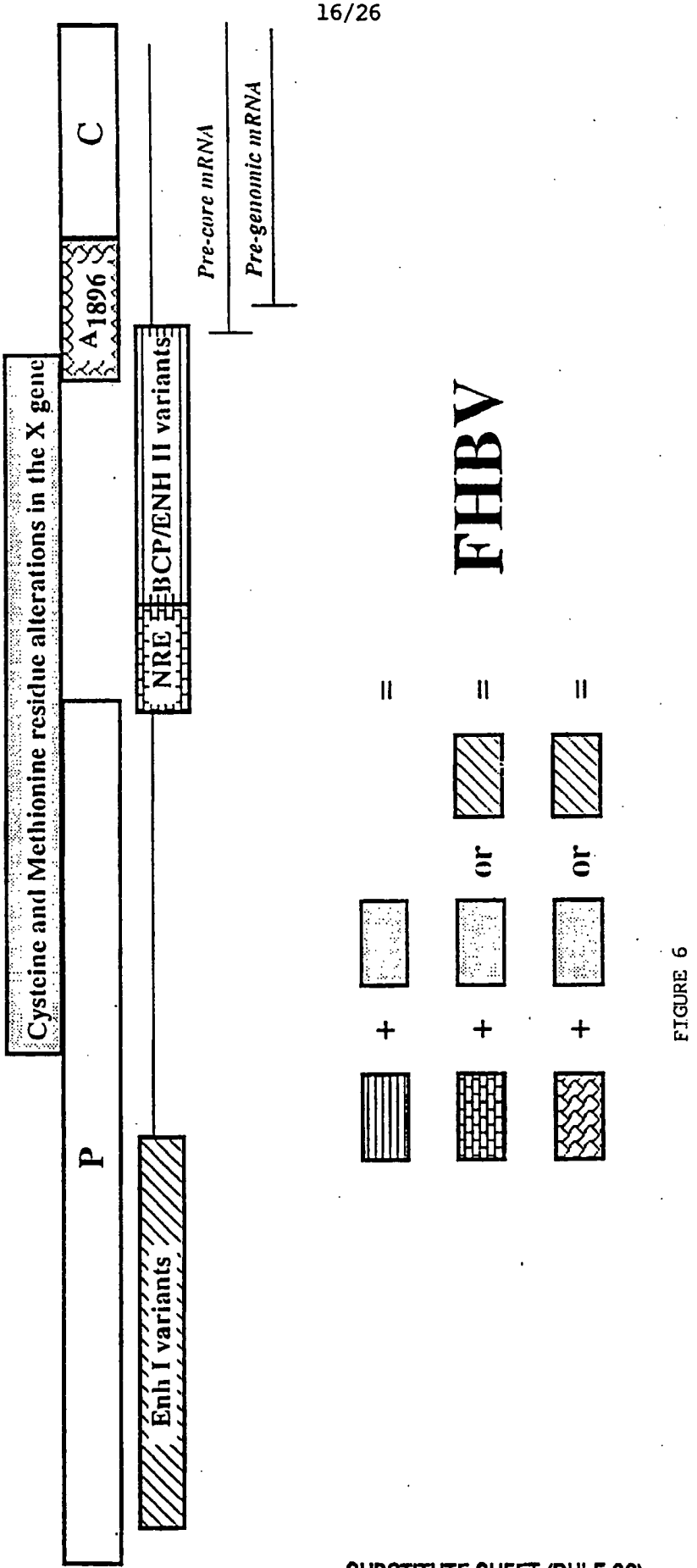

Cysteine and Methionine residue alterations in the X gene
C
A1896
P
Enh I variants
NRE
BCP/ENH II variants
Pre-core mRNA
Pre-genomic mRNA
FHBV
=
=
=
+
+
+
or
or

FIGURE 6

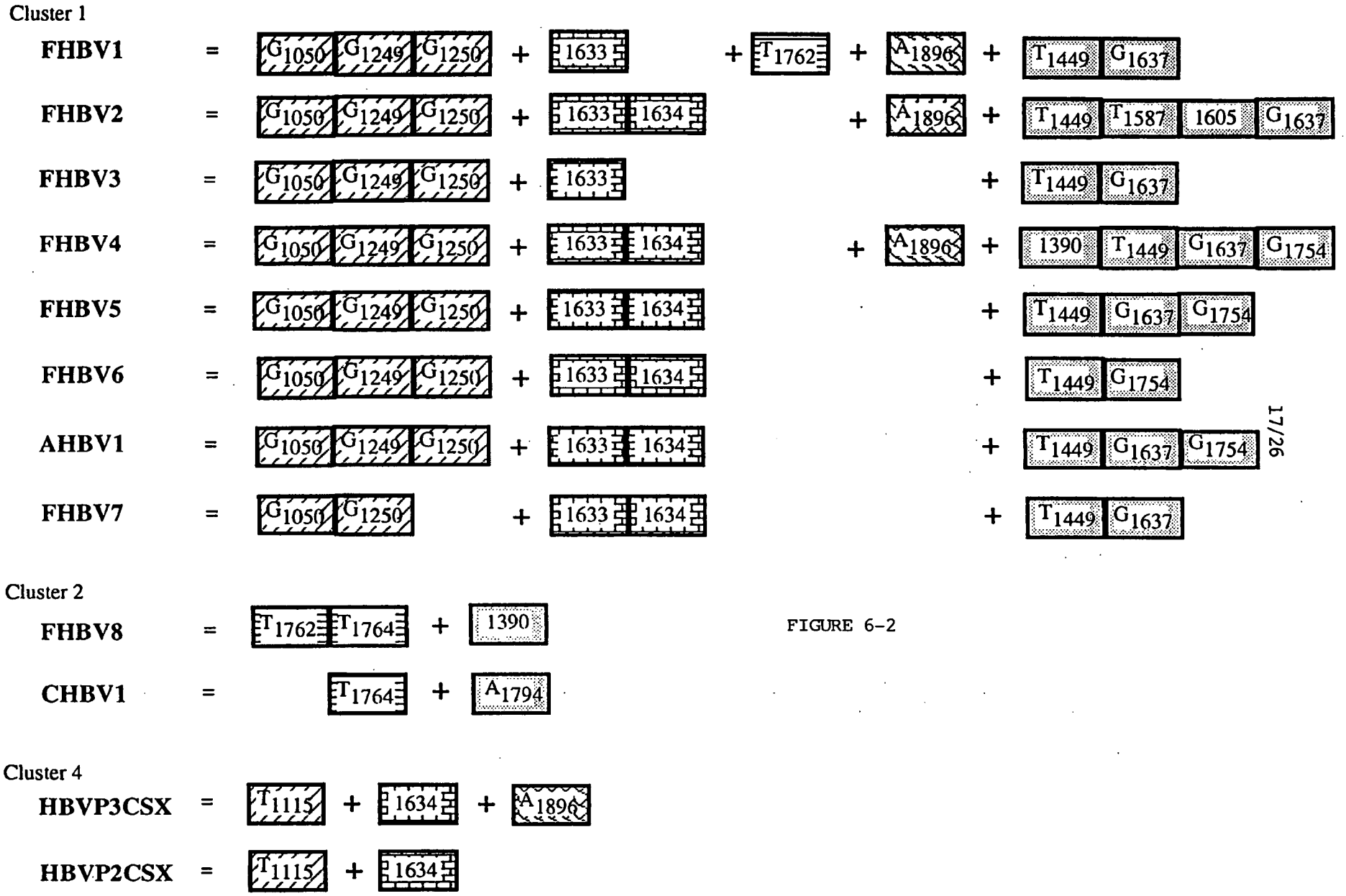
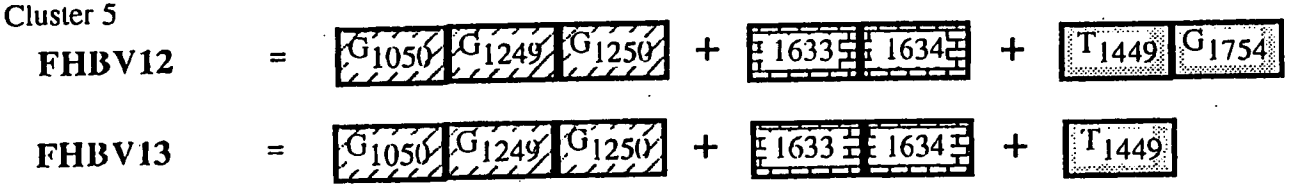
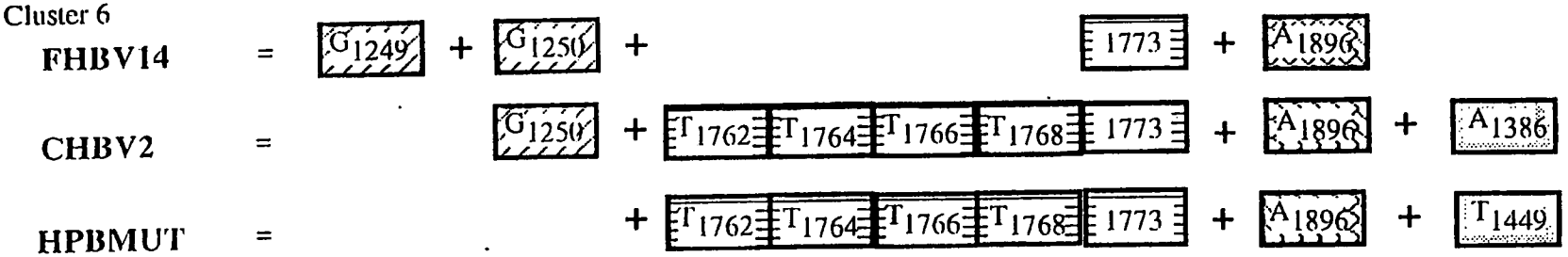

Cluster 1
FHBV1 = G1050 G1249 G1250 + 1633 + T1762 + A1896 + T1449 G1637
FHBV2 = G1050 G1249 G1250 + 1633 1634 + A1896 + T1449 T1587 1605 G1637
FHBV3 = G1050 G1249 G1250 + 1633 + T1449 G1637
FHBV4 = G1050 G1249 G1250 + 1633 1634 + A1896 + 1390 T1449 G1637 G1754
FHBV5 = G1050 G1249 G1250 + 1633 1634 + T1449 G1637 G1754
FHBV6 = G1050 G1249 G1250 + 1633 1634 + T1449 G1754
AHBV1 = G1050 G1249 G1250 + 1633 1634 + T1449 G1637 G1754
FHBV7 = G1050 G1250 + 1633 1634 + T1449 G1637
Cluster 2
FHBV8 = T1762 T1764 + 1390
CHBV1 = T1764 + A1794
Cluster 4
HBVP3CSX = T1115 + 1634 + A1896
HBVP2CSX = T1115 + 1634

FIGURE 6-2

Cluster 5



Cluster 6



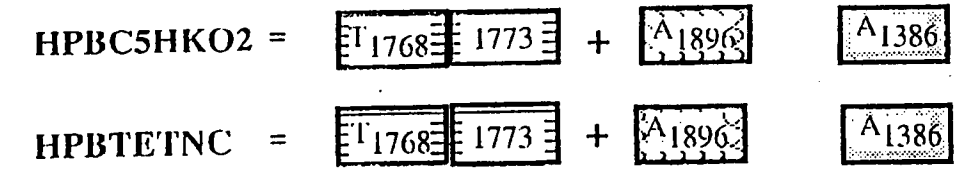
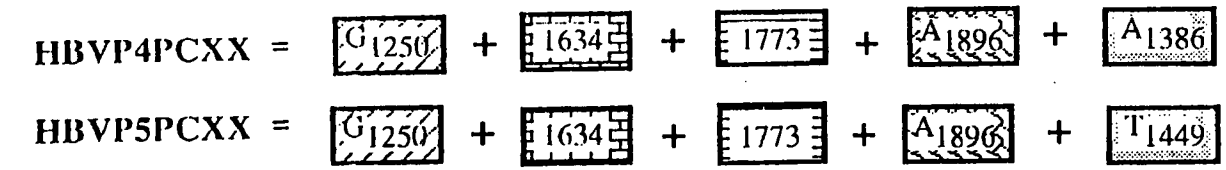


FIGURE 6-3



```
                1643                                                1686
                ↓ (CURS Starts)                                     ↓(Enh-II starts)
Con-ayw         CAAGGTCTTACATAAGAGGACTCTTGGACTCTCTGTAATGTCAACGACCGACCTTGAGGCATACTT
Con-adw         ------------------------------C-A-C----------------------C-----
FHBV-1          -------------------------------C-----------------------------
FHBV-4          ------------------------------AC-----------------------------
FHBV-5          ------------------------------AC-----------------------------
FHBV-8.1        ------------------------------------------------------------
FHBV-8.5        ----------T--------------------C-A-C----------------------T-----
CHBV-1          ------------------------------C-A-C----------------------C-----
FHBV-9          ------------------------------C-A-C----------------------C-----
FHBV-12         ----------A--------------------C-A-C----------------------T-----
FHBV-14         ------------------------------------------------------------
CHBV-2          -------------------------------C-----------------------------
FHBV-15         ------------------------------------------------------------
FHBV-16         ------------------------------C-A-C--------------------------T---

I-40            -------------------------------C-----------------------------
I-59            ---------G-----------------------C-----------------------------
I-69            ---------CG----------------------C-----------------------------
I-89            ----------T----------------------C-----------------------------
I-95            ---------G-----------------------C-----------------------------
I-105           ---------G-----------------------C-----------------------------
I-177           ---------G-----------------------C-----------------------------

                                                    1743
                                                    ↓ (CURS ends, BCP starts)
Con-ayw         CAAAGACTGTTTGTTTAAAGACTGGGAGGAGCTGGGGGAGGAGATTAGATTAAAGGTCTTTGTATT
Con-adw         ----------G-------G----------------------------G-----------------
FHBV-1          -------------------------------T----------------G----T----------C-
FHBV-4          -------------------------------T------------C--------T-A-T-A------
FHBV-5          ------------------G------------T------------C--------T-A-T-A------
FHBV-8.1        -------------------------------T-----------------------T-G--------
FHBV-8.5        ----------G-------G-----------A-------------C---G----T---T--------
CHBV-1          ------------------G-----------------------------G-----------------
FHBV-9          ----------G-------G-----------------------------G-----------------
FHBV-12         ----------G--------------------T----------------G-----------------
FHBV-14         ------------------G------------T------------C----------T-G--------
CHBV-2          ------------------G------------T------------C--------T-A-T-A--C---
FHBV-15         ------------------G------------T-----------C--------------------C-
FHBV-16         ----------G--------------------T-------------------------------

I-40            ----------------------AT------T-------------XXXXXXXXX-A---A------
I-59            ----------C----------------------T-------------------------------C-
I-69            -------------------------------T-------------------------------C-
I-89            -------------------------------T---------------------T-A--------C-
I-95            -------------------------------T---------------------T-A--------C-
I-105           -------------------------------T------------C--------T-A--------C-
I-177           ------------C------------------T-----------------------A--------C-
```

FIGURE 7-1

```
                1776
                ↓(Enh-II ends)
Con-ayw         AGGAGGCTGTAGGCATAAATTGGTCTGCGCACCAGCACCATGCAACTTTTTCACCTCTGCCTAA--TC
Con-adw         ----------------C-----------------------------------------------------
FHBV-1          ------------------------------------------------A-----------------
FHBV-4          ----------------------------------T-----------------------------------
FHBV-5          ----------------------------------T-----------------------------------
FHBV8.1         ------------------------------------------------------------------------
FHBV-8.5        ------------------------------------------------------------------------
CHBV-1          ------------------------------------------------------------------A---
FHBV-9          ------------------------------------------------------------------------
FHBV-12         ------------------C--------------T-----------C---------------------
FHBV-14         ------------------------------------------C--------------A-------
CHBV-2          ----------------------------------T-----------------------------------
FHBV-15         ------------------------------------------------------------------AC--
FHBV-16         ------------------A--------------T--T--------C---------------------

I-40            ------------------------------------------------------------------------
I-59            ------------------------------------------------------------------------
I-69            ------------------------------------------------------------------------
I-89            ------------------------------------------------------------------------
I-95            ----------------------------------T-------------------------------------
I-105           ------------------------------------------------------------------------
I-177           ------------------------------------------------------------------------
```

	1849 ↓(End of BCP)	Genotype	1896	1899
Con-ayw	ATCTCTTGT	D	G	G
Con-adw	---------	A	G	G
FHBV-1	---------	D	A	G
FHBV-4	---------	D	A	A
FHBV-5	--------	D	A	A
FHBV-8.1	----T----	D	A	A
FHBV-8.5	---------	A	G	G
CHBV-1	---------	A	G	G
FHBV-9	---------	A	G	G
FHBV-12	---------	D	G	G
FHBV-14	----T----	D	A	A
CHBV-2	---------	D	A	A
FHBV-15	---------	D	A	G
FHBV-16	---------	A	G	G
I-40	---------	D	G	G
I-59	---------	D	G	G
I-69	---------	D	G	G
I-89	---------	D	A	A
I-95	---------	D	A	A
I-105	---------	D	A	A
I-177	---------	D	A	G

FIGURE 7-2

FIG. 8.A

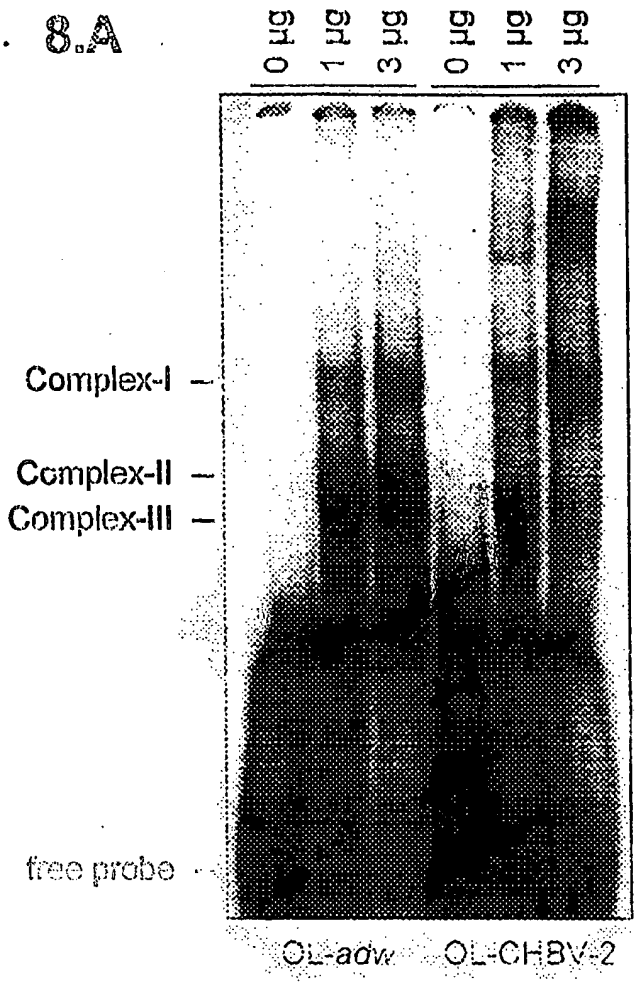

0 µg
1 µg
3 µg
0 µg
1 µg
3 µg
Complex-I
Complex-II
Complex-III
free probe
OL-adw
OL-CHBV-2

FIG. 8.B

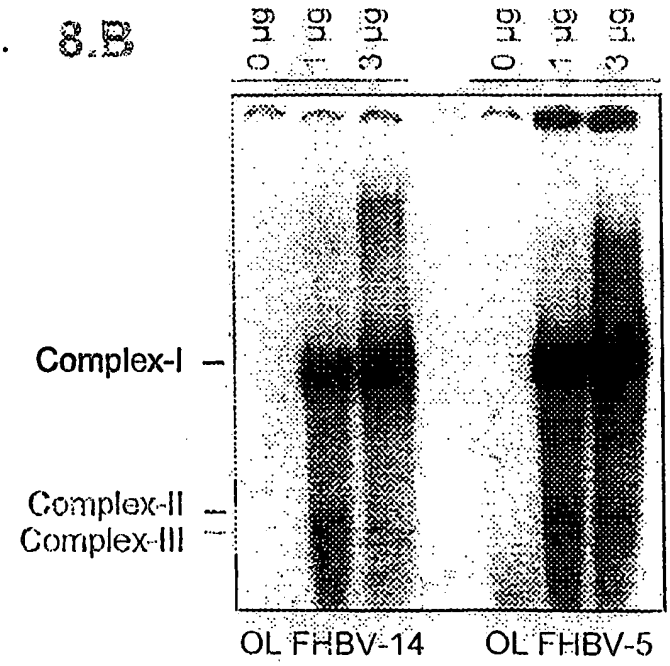

0 µg
1 µg
3 µg
0 µg
1 µg
3 µg
Complex-I
Complex-II
Complex-III
OL FHBV-14
OL FHBV-5

FIG. 8.C

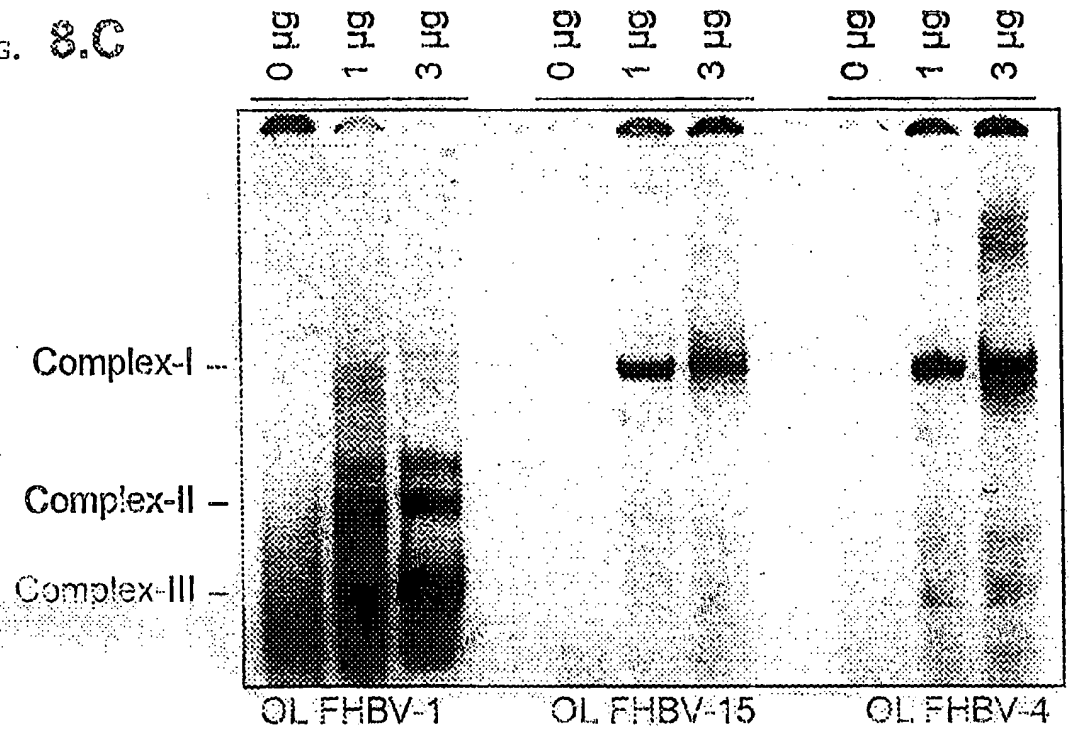

0 µg
1 µg
3 µg
0 µg
1 µg
3 µg
0 µg
1 µg
3 µg
Complex-I
Complex-II
Complex-III
OL FHBV-1
OL FHBV-15
OL FHBV-4

FIG. 8.D

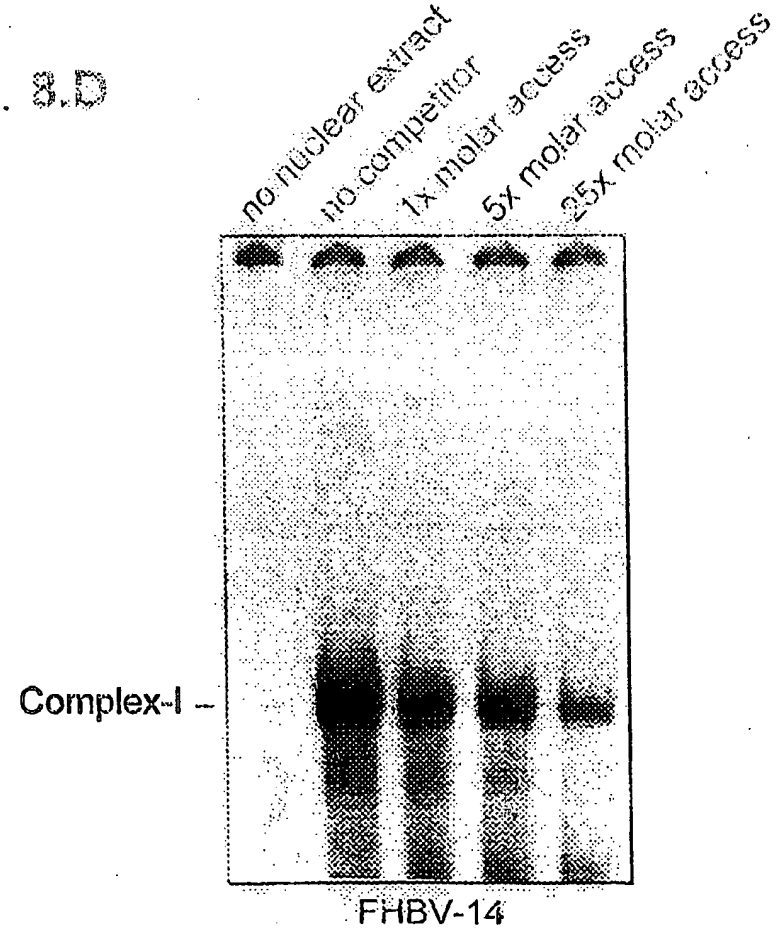

no nuclear extract
no competitor
1x molar access
5x molar access
25x molar access
Complex-I
FHBV-14

FIG. 9A

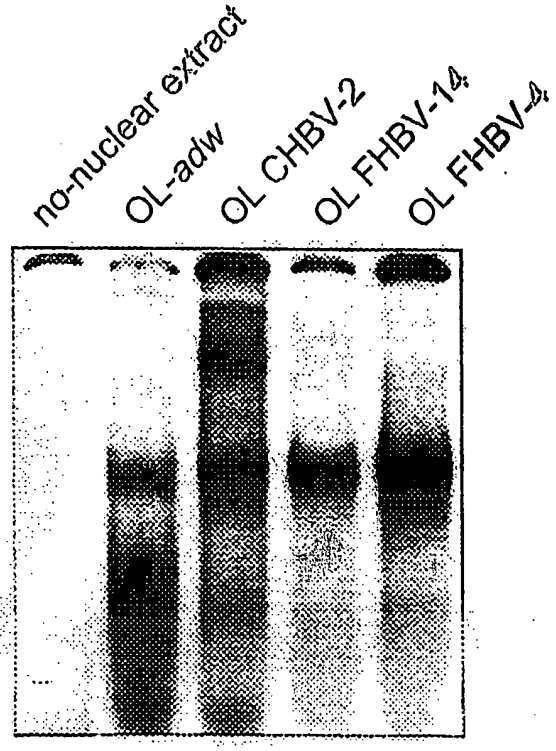
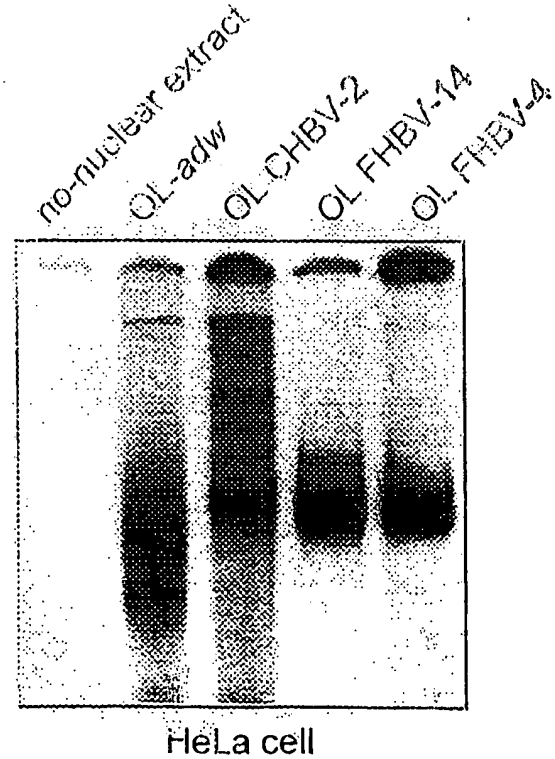

no-nuclear extract
OL-adw
OL CHBV-2
OL FHBV-14
OL FHBV-4
Complex-I
Complex-II
Complex-III
HuH7 cell

FIG. 9B


no-nuclear extract
OL-adw
OL CHBV-2
OL FHBV-14
OL FHBV-4
Complex-I
Complex-II
Complex-III
HeLa cell

Cases	Luciferase value x10^6	Variations in the BCP
Con-*adw*	1.50	
FHBV-1	11.51	T_{1762} A_{1826}
FHBV-4	8.81	C_{1753} A_{1757} T_{1762} A_{1764}, T_{1766} A_{1768} T_{1810}
FHBV-5	10.25	C_{1753} A_{1757} T_{1762} A_{1764}, T_{1766} A_{1768} T_{1810}
FHBV-14	11.39	A_{1757} T_{1764} G_{1766} A_{1834} T_{1845}
CHBV-2	9.68	C_{1753} A_{1757} T_{1762} A_{1764} A_{1768} C_{1771} T_{1810}
FHBV-15	7.98	T_{1678} C_{1752} A_{1757}

FIGURE 10-1

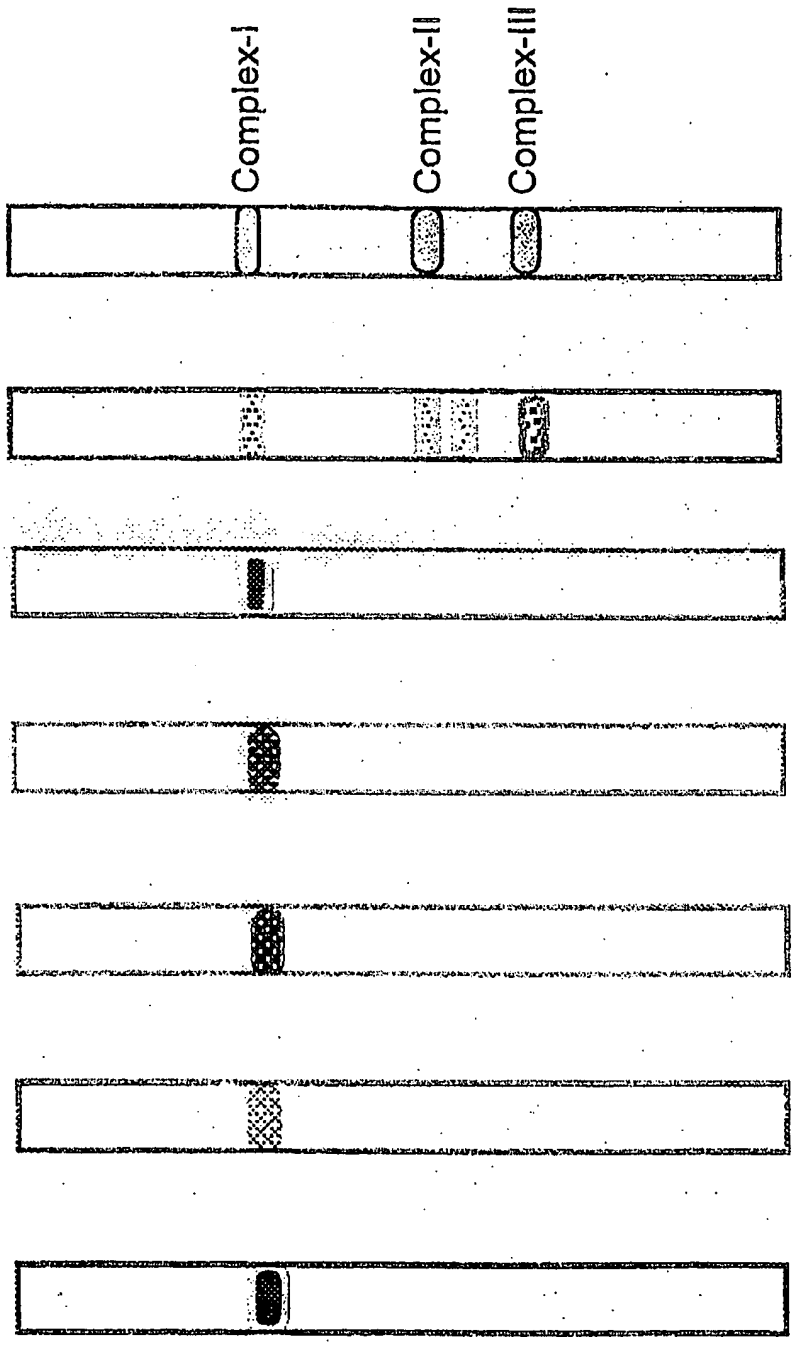


FIG 10-2

FIG. 11